TRANSFORMING GROWTH FACTOR-β AND CILIARY NEUROTROPHIC FACTOR SYNERGISTICALLY REGULATE VASOACTIVE INTESTINAL PEPTIDE THROUGH THE CYTOKINE RESPONSE ELEMENT

2000

PITTS

UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
F. EDWARD HEBERT SCHOOL OF MEDICINE
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

APPROVAL SHEET

Title of Thesis: "Transforming Growth Factor β and Ciliary Neurotrophic Factor Synergistically Regulate Vasoactive Intestinal Peptide Through the Cytokine Response Element"

Name of Candidate: Richard L. Pitts
Neuroscience Program
Master of Science
5 September 2000

Thesis and Abstract Approved:

Aviva Symes, Ph.D. — 9/5/00
Department of Pharmacology — Date
Thesis Advisor

James Coulombe, Ph.D. — 9/5/00
Department of Anatomy & Cell Biology — Date
Committee Member

Ajay Verma, M.D., Ph.D. — 9/5/00
Department of Neurology — Date
Committee Member

Transforming Growth Factor-β and Ciliary Neurotrophic Factor Synergistically Regulate Vasoactive Intestinal Peptide through the Cytokine Response Element

Lee Pitts
Neuroscience Department
Uniformed Services University of the Health Sciences
Bethesda, Maryland

TABLE OF CONTENTS

Abstract

Ciliary Neurotrophic Factor (CNTF) and Transforming Growth Factor-β (TGF-β) are members of two distinct cytokine families that utilize different receptors and signal transduction pathways. Both CNTF and TGF-β can induce transcription of the gene encoding the neuropeptide Vasoactive Intestinal Peptide (VIP) in neuroblastoma cells. CNTF and TGF-β regulate VIP gene transcription through the 180 bp cytokine response element (CyRE) in the VIP promoter. Treatment with both cytokines synergistically stimulates transcription through this element. CNTF rapidly stimulates activation and binding of STAT and AP-1 complexes to the CyRE. TGF-β induces the translocation of a smad protein complex into the nucleus. Neither pathway was observed to influence the signal transduction cascade of the other. Two smad binding sites were found in the CyRE, by sequence similarity and binding of purified smad proteins. These sites bound purified smad proteins and mutation of these two smad binding elements in the CyRE diminished TGF-β induction of VIP CyRE transcription. Thus synergy between CNTF and TGF-β signaling pathways may be mediated by formation and binding to the CyRE of a complex consisting of TGF-β activated smads and CNTF-stmiulated STAT and AP-1 proteins.

Introduction

Ciliary Neurotrophic Factor and CNTF Regulation of VIP

Ciliary Neurotrophic Factor (CNTF) is a member of the IL-6 family of cytokines. Members of this family include Interleukin-6 (IL-6), Leukaemia Inhibitory Factor (LIF; Gearing, et al., 1987), Growth Promoting Activity (GPA; Leung, et al., 1992), Cardiotrophin-1 (CT-1; Pennica, et al., 1995), Oncostatin M (OSM; Malik, et al., 1989), and CNTF (Barbin, et al., 1984; Lin, et al., 1989). These cytokines are related because they share similar tertiary structure, receptors, and signaling pathways (Bazan, 1991; Gearing and Bruce, 1992; Gearing, et al., 1992; Ip, et al., 1992). All members utilize a common transmembrane receptor component, gp130 (Reviewed in Murphy, et al., 1997). Often these cytokines are referred to as the 'gp130 cytokines.'

CNTF induces transcription of multiple neuropeptide genes in a neuroblastoma cell line, NBFL (Symes, et al., 1993). CNTF regulation of vasoactive intestinal peptide (VIP) has been well characterized (Symes et al., 1993, 1994, 1995, 1997, 2000; Baumann, et al., 1994). A region of the VIP promoter that was necessary and sufficient for CNTF-mediated gene transcription was mapped to a 180 bp element, the Cytokine Response Element (CyRE) (Symes, et al., 1994). CNTF stimulation leads to activation and binding of STAT1/STAT3 complexes to the CyRE within 15 minutes (Symes, et al., 1994). CNTF stimulation also induced the binding of AP-1 complexes consisting of c-Fos, JunB, and JunD to an AP-1 site in the CyRE within 1 hour (Symes, et al., 1997). Both AP-1 and STAT sites are critical for CNTF-mediated VIP gene transcription.

CNTF transduces signals through a receptor complex consisting of three components: LIFR-β, gp130, and CNTFR-α (Davis, et al., 1991; Stahl and Yancopoulos, 1994). CNTF binds to CNTFR-α and recruits LIFR-β and gp130 (Stahl and Yancopoulos, 1994; Sleeman, et al., 2000). LIFR-β and gp130 both contain extracellular, transmembrane, and cytoplasmic domains where as CNTFR-α is a GPI-linked component, containing only an extracellular domain (Ip, et al., 1993; Stahl and Yancopoulos, 1994). LIFR-β and gp130 have no intrinsic kinase activity but constitutively associate with Janus kinases (JAKs) (Stahl, et al., 1994). Dimerization leads to the transphosphorylation of the JAK kinase (Liu, et al., 1997; Gauzzi, et al., 1996; Feng, et al., 1997). JAKs then phosphorylate multiple tyrosine residues on the cytoplasmic tail of each receptor subunit (Stahl, et al., 1994; Sleeman, et al., 2000). These phosphotyrosine resides then become docking sites for a class of transcription factors called Signal Transducer and Activator of Transcription (STATs). Once docked, STATs are phosphorylated on specific serine and tyrosine residues (Sleeman, et al., 2000). These activated STATs dissociate from the receptor, dimerize, and translocate to the nucleus where they bind specific recognition elements in DNA (Reviewed in Liu, et al., 1998; Segal and Greenberg, 1996; Heim, 1999; Imada and Leonard, 1999). CNTF stimulates the activation and formation of Activator Protein-1 (AP-1) DNA-binding complexes (Symes, et al., 1997). CNTF also stimulates PI3-kinase and MAP kinase pathways through activation of PLC-γ, PI3-kinase, Shc, Grb2, Raf-1, Erk1 and Erk2 (Boulton, et al., 1994). Thus CNTF-mediated gene transcription can potentially be influenced by multiple signal transduction cascades.

Transforming Growth Factor – β

TGF-β is a member of the Transforming Growth Factor – β superfamily. Other members of this family include Activin, Bone Morphogenic Protein (BMP), inhibin, Growth and Differentiation Factor (GDF), Mullerian Inhibitory Substance (MIS), and a distantly related Glial Cell Line-Derived Neurotrophic Factor (GDNF). Most family members signal through a multi-component receptor complex containing intrinsic serine/threonine kinase activity (Reviewed in Massague, 1998). TGF-β binds to a type II receptor subunit (TβR-II) (Laiho, et al., 1990; Boyd and Massague, 1989). Once bound, the ligand-TβRII complex recruits the type I subunit (TβR-I) (Franzen, et al., 1993). The TβR-II phosphorylates the TβR-I in a transmembrane domain that is rich in glycine and serine (GS domain) (Souchelnytskyi, et al., 1996; Wrana, et al., 1994; Wieser, et al., 1995). Once phosphorylated, the TβR-1 is 'activated.' Activated TβR-1 subsequently phosphorylates a newly identified class of transcription factors, smad proteins (Reviewed in Massague, 1998).

Smad proteins are downstream components of receptor activated TGF-β signaling. Nine distinct isoforms of smads have been characterized. These fall into three classes: Receptor-regulated smads (R-smads), Common smads (Co-smads), and Inhibitory smads (I-smads). Smads 1, 2, 3, 5, and 8 are referred to as R-smads. Smad 4 is a ubiquitously expressed Co-smad. Smad 6 and 7 are referred to as I-smads as they interfer with smad activation and dimerization. R-smads and Co-smads share sequence similarity between their amino terminal (MH1) and carboxy terminal (MH2) domains. A less similar linker region separates these two domains. A membrane-associated protein, SARA (Smad Anchor for Activation) binds non-phosphorylated forms of R-smads and

presents them to the activated TβR-I (Tsukazaki, et al., 1998). Activated TβR-I phosphorylates R-smads on specific serine residues in a carboxy SSXS motif. Activated R-smads dissociate from the receptor and from SARA. Phosphorylated R-smads then form homo-oligomers and hetero-oligomers with each other and with smad 4, a Co-smad. Smad complexes translocate to the nucleus where they influence gene transcription through either direct DNA-binding or protein-protein interactions with other DNA-binding proteins (Reviewed in Zhang and Derynk, 1999; Padgett, et al., 1998; Kretzschmar and Massague, 1998; Derynck, et al., 1998; Attisano and Wrana, 2000; ten Dijke, et al., 2000). This interaction serves either as an activator or repressor of gene transcription.

Smad regulation of transcription

Smads, once activated, can participate in both transcriptional activation and transcriptional repression. Smads can recruit the assembly of coactivators of transcription to promoter regions. A general feature of transcriptional activation is the recruitment of histone acetyl transferases (HATs). HATs acetylate the amino-terminal tails of histones and reduce DNA coiling around histones. This allows for greater access to DNA by transcription factors. Both CREB-Binding Protein (CBP) and p300 have intrinisic histone acetyl transferase activity (Bannister, et al., 1996; Ogryzko, et al., 1996). Overexpressed CBP enhances TGF-β mediated transcription of the plasminogen activator inhibitor-1 (PAI-1) promoter in endothelial cells (Topper, et al., 1998). Immunoprecipitation of either Smad proteins or CBP/p300 revealed a physical interaction between these two proteins in 293T and HaCaT cells (Pouponnet, et al., 1998). Interaction between Smad 3 and CBP is mediated through the carboxy terminus of Smad

3, the region required for smad activation (Feng, et al., 1998). Overexpression of p300 also enhances Smad 3 and Smad 4 dependent transcription of a luciferase reporter construct driven by consensus Smad 3 and Smad 4 binding sites (SBE4-Luc) (Janknecht, et al., 1998). E1A, an adenoviral oncoprotein, inhibits TGF-β mediated transcriptional activation by competing with Smad proteins for binding to CBP/p300 (Nishihara, et al., 1999). Disrupting the formation of the coactivator complexes containing HAT activity leads to inhibition of transcription. Thus TGF-β activated smad proteins translocate to the nucleus where they bind to coactivators such as CBP/p300 to activate transcription (Topper, et al., 1998; Pouponnet, et al., 1998; Feng, et al., 1998; Janknecht, et al., 1998).

Recent evidence suggests Smad proteins are possible negative regulators of transcription by recruiting corepressors and associated proteins (Verschueren, et al., 1999; Wotton, et al., 1999; Nomura, et al., 1999). A general feature of transcriptional repression is the recruitment of histone deacetylases (HDACs). HDACs allow tighter coiling of DNA around histones and thus limit access of transcription factors to DNA. In this manner, repressors and corepressor either downregulate active genes or maintain genes in an inactive state. TGIF is a protein implicated in Smad-mediated repression. Smad 2, through its interaction with TGIF, is able to recruit HDAC1 and thus repress transcriptional activation of TGF-β responsive promoters (Wotton, et al., 1999). Ski, a nuclear oncoprotein, was also demonstrated to associate with smads in repressing transcription (Nomura, et al., 1999; Luo, et al., 1999). The formation of a transcriptional repressor complex is most likely the reason for ski repression of the TGF-β responsive 3TP-Lux reporter (Luo, et al., 1999). Thus, multiple corepressors may be involved in repressing TGF-β mediated transcription.

Specific Aims

Given that CNTF and TGF-β are both regulators of neuropeptide expression, we hypothesize that they might interact in regulating VIP expression in NBFL cells. Although CNTF-regulation of VIP is well characterized, involvement of TGF-β in VIP regulation has not been described. Despite what is already known about CNTF and TGF-β mediated signal transduction, interaction between these two signaling pathways in gene regulation is poorly understood. Here we investigate the mechanisms by which CNTF and TGF-β regulate VIP gene expression. We sought to ascertain whether TGF-β regulates VIP gene expression and through which signal transduction pathways this regulation may be achieved. We also sought to investigate whether there was any cross-talk between known CNTF and TGF-β signaling pathways. Finally we tried to determine the composition of any transcriptional complexes induced in TGF-β regulation of VIP.

Figure 1. CNTF signaling in NBFL cells

CNTF activates signal transduction cascades through a trimeric receptor complex. CNTF binds to a GPI-linked receptor component, CNTFR-α. Binding of CNTF to CNTFR-α initiates recruitment and dimerization of LIFR-β and gp130. LIFR-β and gp130 have no intrinsic kinase activity but are associated with the tyrosine kinase, JAK. Dimerization leads to transphosphorylation of JAKs and subsequent phosphorylation of tyrosine residues along the cytoplasmic tails of LIFR-β and gp130. These phosphotyrosines become docking sites for STAT proteins. STATs bind and undergo phosphorylation by JAKs. Phosphorylated STATs dissociate from the receptor, dimerize, and translocate to the nucleus where they bind a specific recognition element (P3) in the CyRE. CNTF stimulation also leads to the activation and formation of AP-1 complexes. The formation of this complex is most likely nuclear. AP-1 proteins also bind to a specific region (P2) of the CyRE. Both STAT and AP-1 components of CNTF signaling are important for CNTF regulation of VIP transcription through the CyRE.

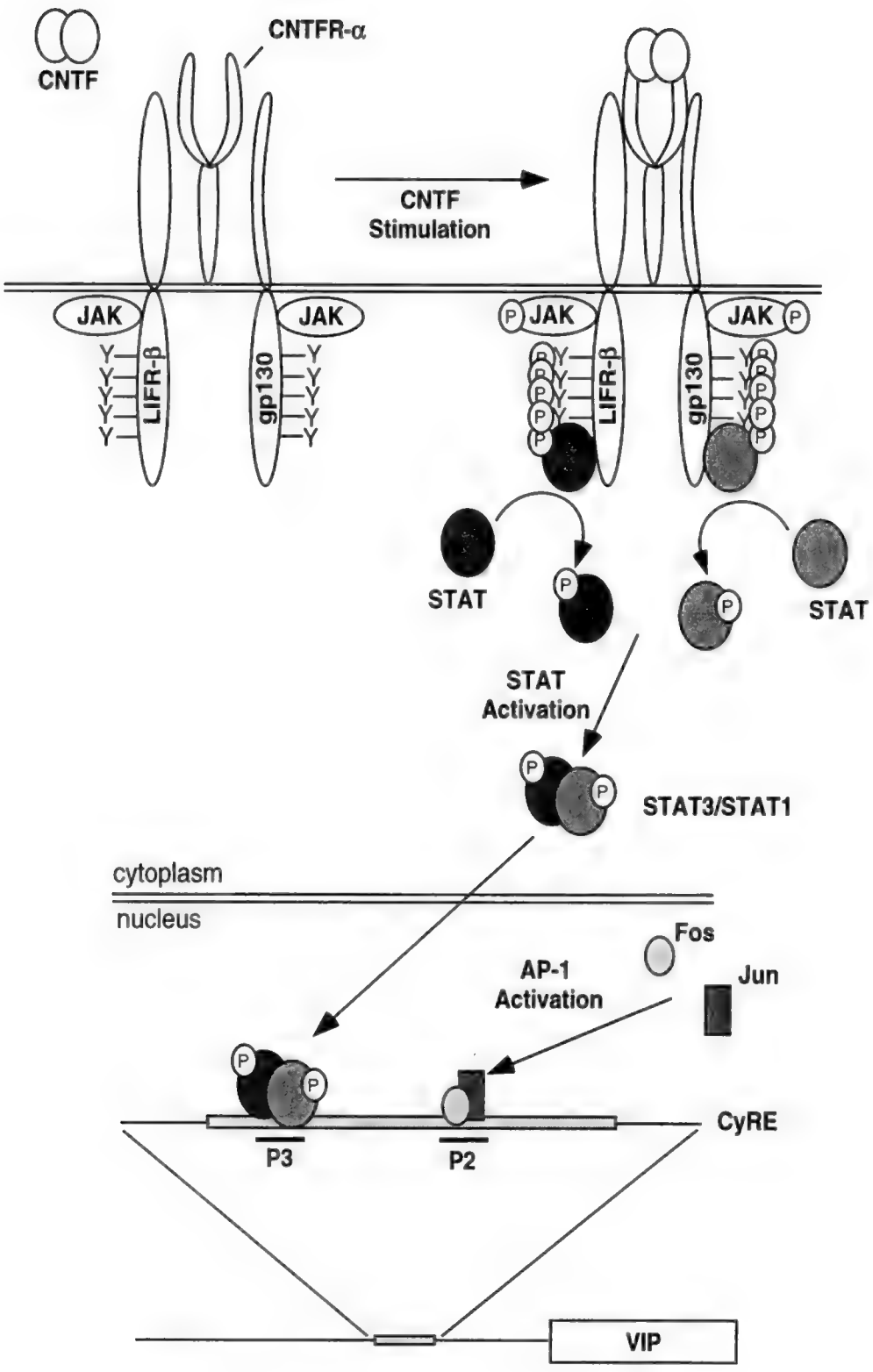
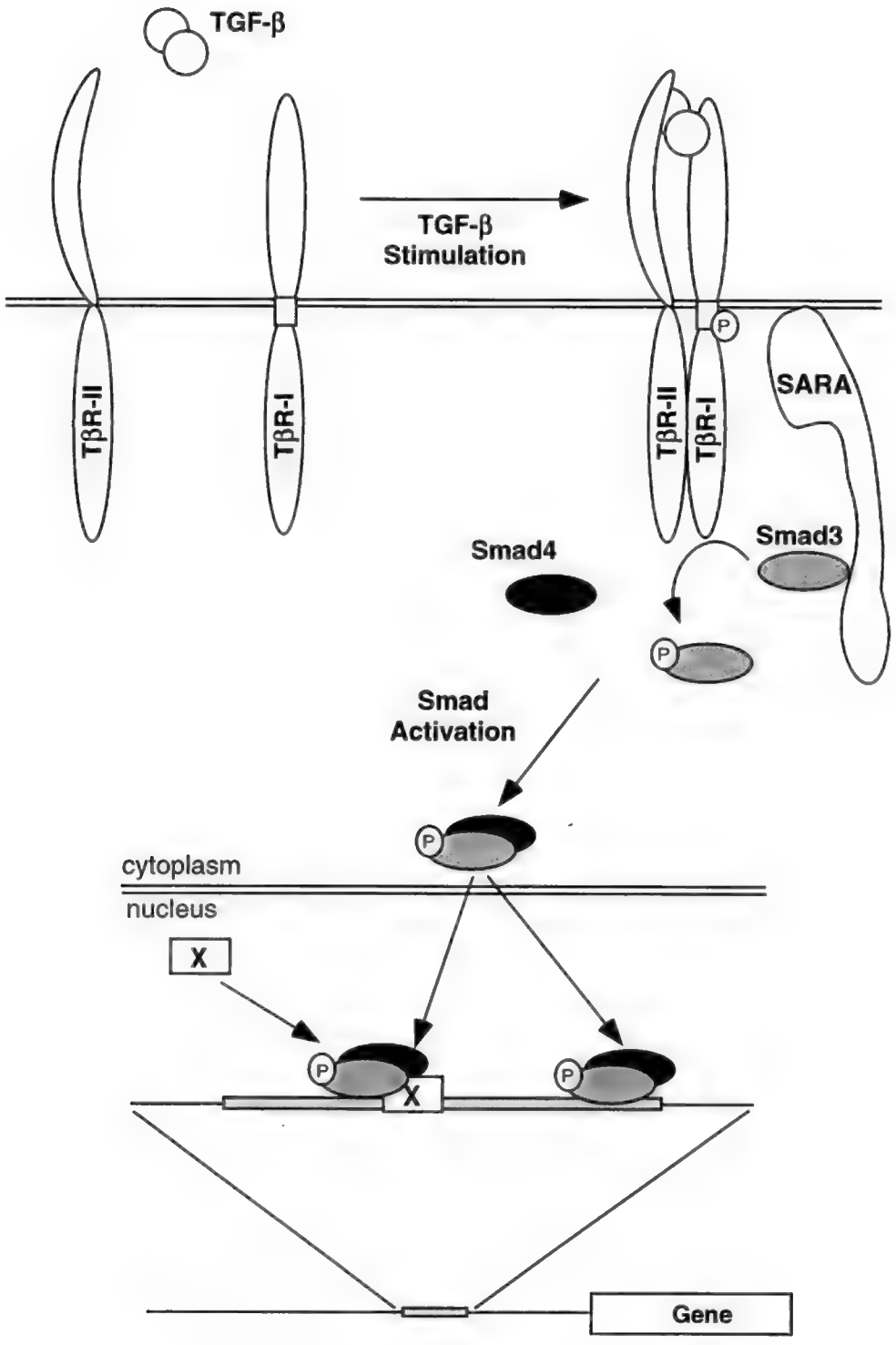

CNTF
CNTFR-α
CNTF
Stimulation
JAK
LIFR-β
gp130
JAK
P
JAK
LIFR-β
gp130
JAK
P
STAT
P
P
STAT
STAT
Activation
P
P
STAT3/STAT1
cytoplasm
nucleus
Fos
AP-1
Activation
Jun
P
P
CyRE
P3
P2
VIP

Figure 2. TGF-β signal transduction

TGF-β initiates a signal transduction cascade through a pair of serine/threonine kinase receptor components. TGF-β binds to a type II receptor component (TβR-II). Binding of TGF-β to TβR-II results in recruitment and dimerization of TβR-II with a type I receptor component (TβR-I). Both receptor components contain endogenous serine/threonine kinase activity. Dimerization of TβR-II with TβR-I leads to phosphorylation of TβR-I in a glycine/serine rich domain called the GS box. Activation of TβR-I by phosphorylation enables the phosphorylation of downstream transcription factors, smads. A membrane associated protein, SARA, presents smad proteins to activated TβR-I for phosphorylation. Phosphorylated smads dissociate from TβR-I and SARA and dimerizes with a Co-smad, Smad 4. Smad protein complexes translocate to the nucleus where they bind other DNA-binding proteins or DNA directly to regulate TGF-β mediated transcription.


TGF-β
TGF-β
Stimulation
TβR-II
TβR-I
TβR-II
TβR-I
P
SARA
Smad4
Smad3
P
Smad
Activation
P
cytoplasm
nucleus
X
P
X
P
Gene

Materials and Methods

Materials

Cell culture reagents were obtained from Mediatech (Herndon, VA), fetal bovine and horse serum from Life Technologies, Inc. (Gaitherburg, MD), and culture plates from Costar (Corning, NY). Human Transforming Growth Factor-β-1 was purchased from R&D Systems, Inc. (Minneapolis, MN). Recombinant Human CNTF was a gift from Regeneron Pharmaceuticals (Tarrytown, NY). Polyclonal Smad 2/3 antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). pGE5-Luc and associated GAL4 plasmids were a generous gift of Dr. Robert J. Lechleider.

Cell Culture

NBFL neuroblastoma cells were maintained in DMEM (4.5 g glucose/liter) (Mediatech) supplemented with 5% (vol/vol) fetal bovine serum, 5% horse serum, and 1% glutamine (Life Technologies, Inc.) in a humidified incubator (5% CO_2, 37°C).

Nuclear Protein Extraction

NBFL cells were plated in 10 cm dishes and allowed to reach 90% confluency, before cytokine treament. At specific times, treated cells were harvested. Cells were rinsed with 1X cold phosphate buffered saline (PBS), scraped into 1 ml of PBS, and centrifuged for 15 seconds at 8,000 rpms. The supernatant was removed and cell pellets were resuspended in 400 µl of cold Buffer A (10 mM HEPES [pH 7.9], 10mM KCl, 0.1mM EDTA and EGTA, 1mM DTT, 1mM PMSF, 100µM Na_3VO_4, 10 µg/ml Leupeptin, 10

μg/ml Aprotinin, and 1 μg/ml Pepstatin). Cells were placed on ice to swell for 15 minutes. Cells were lysed with 25μl of 5% Nonidet P-40, centrifuged for 30 seconds at 14,000 rpms, and the supernatant removed. The nuclear pellet was resuspended in 60 μl of cold Buffer C (20mM HEPES [pH 7.9], 0.4M NaCl, 1mM EDTA and EGTA, 1mM DTT, 1mM PMSF, 100μM Na_3VO_4, 10 μg/ml Leupeptin, 10 μg/ml Aprotinin, and 10 μg/ml Pepstatin) and shaken gently at 4°C for 15 minutes. Samples were centrifuged at 14,000 rpm for 5 minutes at 4°C. Cleared nuclear extracts were aliquoted and stored at –80°C until needed.

Cellular Lysate Preparation

NBFL cells were plated in 10 cm dishes and treated with cytokines as indicated. Cells were washed twice in 1X cold PBS containing 1mM Na_3VO_4, harvested in 500 μl of harvesting buffer (1X TBS, 1% (vol/vol) nonidet-P40, 1mM PMSF, 1mM Na_3VO_4, 10μg/ml leupeptin, 10μg/ml aprotinin, and 1μg/ml pepstatin) and rotated at 4°C for 10 minutes to complete lysis. Cell lysates were centrifuged at 14,000 rpms for 10 minutes at 4°C. Cleared lysates were collected and stored at –80°C until needed.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts (2 μl) were incubated for 15 minutes on ice with 0.5 ng radiolabeled probe in a final 20 μl binding reaction (2mM potassium phosphate buffer [pH 7.8], 8mM HEPES [pH 7.9], 40 mM KCl, 280 μM EDTA, 8% glycerol, 1.2 mM DTT, and 0.1 mg/ml poly-dI/dC). When unlabeled oligonucleotides were used for competition, 50 ng of oligonucleotide (100X) was added to the sample and incubated on ice 10 minutes prior

to incubation with the probe. Samples were loaded onto a 5% non-denaturing polyacrylamide gel and subjected to electrophoresis at 200 volts for 3 hours at 4°C. Gels were dried and exposed to film at –80°C for appropriate exposure times.

Radiolabeled Oligonucleotides (for EMSA)

Complementary oligonucleotides were synthesized with 3' GGG overhangs. Sequences of oligonucleotides are shown in Table 1. Both strands (100 μg each) were boiled in annealing buffer (200mM sodium phosphate buffer [pH 7.4], 10 mM EDTA, and 1M KCl) for 5 minutes and allowed to cool slowly overnight from 85°C to 25°C to anneal. Annealed oligonucleotides (100 ng) were radiolabeled using MMLV reverse transcriptase (Pharmacia) and [α-^{32}P]-dCTP for 15 minutes at 37°C. Radiolabeled oligonucleotides were purified on G25 spin columns (Boehringer Mannheim) and stored at –20°C.

RNA Isolation

RNA was isolated by phenol/chloroform extraction on ice. Cells were trypsinized and pelleted at 2,500 rpm for 5 minutes. Cell pellets were placed on ice and lysed in 1 ml of NP-40 lysis solution (50mM Tris [pH 8.0], 100mM NaCl, 5mM $MgCl_2$, and 0.5% (vol/vol) nonidet-P 40). Cells were centrifuged at 2,500 rpms for 5 minutes at 4°C. Lysate (500 μl) was added to an equal volume of equilibrated phenol (500 μl) and 0.1% SDS. Samples were vortexed and centrifuged at 13,000 rpm for 3 minutes at 4°C. 450 μl of the aqueous phase was transferred to an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) (450 μl). Samples were vortexed and centrifuged at 13,000 rpm for 3 minutes at 4°C. The aqueous phase (400 μl) was transferred to an equal volume of

chloroform/isoamyl alcohol (24:1) (400 µl). Samples were vortexed and centrifuged at 13,000 rpm for 3 minutes at 4°C. The aqueous phase was transferred to a fresh tube and RNA precipitated by the addition of 2.5 volumes of cold 100% ethanol and 0.3M sodium acetate. Samples were inverted several times and placed at –80°C for 30 minutes. Samples were centrifuged at 13,000 rpm for 15 minutes at 4°C. Ethanol was aspirated off and RNA pellets were washed in cold 70% ethanol (600 µl). The remaining ethanol was carefully aspirated off and pellets were allowed to dry. Pellets were resuspended in 100 µl of DEPC water and concentrations determined by spectrophotometer optical density analysis at 260 nm.

Northern Blot Analysis

RNA (10 µg) was dried down and resuspended in 9 µl of northern loading buffer (1X MOPS buffer, 16.6% (vol/vol) formaldehyde, and 50% (vol/vol) formamide). Samples were incubated at 65°C for 15 minutes. Loading dye (10X) (1µl) was added to each sample and samples were loaded onto a formaldehyde/agarose gel (1X MOPS buffer, 1.3% agarose, and 17% formaldehyde). The gel was subjected to electrophoresis at 200 volts in 1X MOPS for 2-3 hours at room temperature. RNA was transferred to nylon membranes (Genescan, NEN) by electroblotting. RNA was cross-linked to the nylon membrane by UV light (Stratalinker 1800, Stratagene). Membranes were soaked and rinsed once in 2X SSC. Membranes were prehybridized in 10 mls of hybridization buffer (50% formamide, 20% dextran sulfate, 5% SDS, 0.2% polyvinyl pyrrolidone, 0.2% BSA, 0.2% ficoll, 0.1% sodium pyrophosphate, 1M NaCl, 0.05M Tris-HCl [pH 7.5], and 10µg/ml salmon sperm) at 42°C for 2 hours. A northern probe was boiled at 95°C for 10

minutes and 200 µl of probe was added to the hybridization buffer. Membranes were hybridized overnight at 42°C while rotating. Blots were washed initially in 2X SSC with 0.1% SDS at 65°C for 15 minutes, followed by washing in 0.2X SSC with 0.1% SDS at 65°C for 30-60 minutes. Blots were exposed to film at –80°C with intensifying screens.

Northern Blot Probe

Double stranded DNA fragments (25 ng) were radiolabeled with an Oligolabeling Kit using Klenow (Pharmacia) at 37°C for 60 minutes. Radiolabeled fragments were separated from unincorporated nucleotides through a NICK column (Pharmacia) and stored at –20°C until needed.

Bradford Protein Assay

All nuclear and cellular lysate extracts were quantitated for protein content using a Bradford Protein Assay. Samples (2 µl) were incubated with 20% protein dye (BioRad) for 5 minutes. Samples were analyzed on a spectrophotometer at a fixed wavelength of 595 nm.

Calcium Phosphate Transient Transfections

Cells were plated in 6-well plates at a density of $1.5x10^5$ cells/ml one day before transfection. Reporter plasmids (2-3 µg) and β-galactosidase control plasmids (1µg) were mixed in a solution containing 5mM $CaCl_2$ and 0.1X HEPES Buffered Saline (HBS). The calcium phosphate-DNA precipitate was incubated with cells overnight. The cells were rinsed twice with DMEM and 1 ml serum-free medium was placed in each

well for approximately 8 hours. The cells were treated with cytokines in serum-free medium for approximately 36 hours. The cells were rinsed in PBS and lysed in 200 µl of lysis solution (Galacto-Light Plus Kit). Lysates were subjected to centrifugation at 14,000 rpms for 10 minutes then assayed for luciferase and β-galactosidase activity.

Luciferase and β-galactosidase Assays

Lysates (100 µl) were incubated with 368 µl of a reaction solution (25mM glyclglycine [pH 7.8], 15mM $MgSO_4$, 2.4mM EGTA, 12mM potassium phosphate buffer, 0.8mM DTT, and 0.8mM ATP). Luciferin (100 µl of 1X) was autoinjected by an Dynex luminometer and relative light units (RLU) were recorded. To normalize luciferase values, lysates were assayed for a constitutively expressed enzyme, β-galactosidase, using a Galacto-light Plus Kit (Tropix, Inc., MA) according to manufacturer's instructions. Samples (20 µl) were heat inactivated at 48°C for 30 minutes. Galacton-Plus Substrate was diluted 1:100 with Galacto-light Reaction Buffer Diluent. Diluted substrate (100 µl) was added to each tube and 10 µl of heat inactivated sample was added in 30 second intervals. Samples were allowed to incubate for 1 hour and RLU levels monitored with an Dynex luminometer in 30 second intervals. Luciferase data was normalized to β-galactosidase activity to control for transfection efficiency in each well.

Western Blotting

Samples were denatured in 1X Sample Buffer containing 125mM DTT by boiling for 5 minutes. Samples were loaded onto a Tris-Glycine gel (12% acrylamide/bis, 0.38M Tris-HCl [pH 8.8], 0.1% SDS, and 0.1% APS) and subjected to electrophoresis at 200 volts

for 1 hour at room temperature. The gel was then transferred to nitrocellulose membranes at 100 volts for 1 hour at 4°C. Membranes were blocked for 1 hour in PBS with 5% non-fat dry milk at room temperature. Membranes were incubated with primary antibody diluted 1:1000 in PBS with 5% milk for 1 hour at room temperature while shaking gently. Primary antibody was rinsed off with a series of four 5 minute washes in PBS with 0.1% Tween-20. Membranes were incubated with secondary antibody diluted 1:2000 in PBS with 5% milk for 1 hour at room temperature while shaking. Excess secondary antibody was removed by four 5 minute washes in PBS with 0.1% Tween-20. A final 10 minute wash in PBS was used to remove excess detergent from the blot. Blots were detected with an ECL-Plus (Amersham) kit following manufacturer's instructions.

GST Fusion Protein Preparation

Single colonies of transformed E. coli. containing expression plasmids for GST-Smad 4 and GST-Δc Smad 3 were picked and grown in 5 mls Luria Broth with 1 mg/ml ampicillin overnight shaking at 37°C. Bacterial cultures (1 ml) were added to 50 mls Luria Broth with 1 mg/ml ampicillin and grown at 37°C while shaking until they reached mid-log phase. Mid-log phase was determined by an OD of 0.5 to 0.8 at 595 nm. Mid-log phase cultures were treated with 100 mg/ml IPTG for 4 hours while shaking at 37°C to stimulate the expression of the GST proteins. Bacterial cultures were pelleted by centrifugation at 8,000 x g at 4°C for 5 minutes. Pellets were washed in 3 mls STE buffer (25mM Tris-HCl [pH 8.0], 150mM NaCl, and 1mM EDTA) and centrifuged at 8,000 x g at 4°C for 5 minutes. Pellets were resuspended in 3 mls STE buffer containing 100 μg/ml lysozyme and placed on ice for 15 minutes. Protease inhibitors were added to the

resuspended cultures (3 μg Pepstatin, 15 μg Leupeptin, 30 μg Aprotinin, and 100μM PMSF). Sarkosyl (530 μl) was added (1.5% final concentration) and cultures were vortexed. Cells were sonicated on ice in 15 to 30 second intervals for 2 minutes to avoid heating the proteins. Lysed cells were centrifuged at 8,000 x g at 4°C for 5 minutes. Supernatant was transferred to a new tube and Triton-X 100 (875 μl) was added. Sample was vortexed for 10 second. Samples (1.5 mls) were added to glutathione-agarose beads (250 μl), vortexed, and incubated for 1 hour at 4°C while rotating. Samples were centrifuged at 3400 rpm at 4°C for 5 minutes. Supernatant was removed and beads were washed 5 times and centrifuged at 3400 rpm at 4°C for 5 minutes each time. Pellets were resuspended in 3 mls of Glut-Elute (2.5mM Tris [pH 8.0], 10mM glutathione, and 0.01% Triton-X 100) [pH 7.4]. Samples were incubated at 4°C for 30 minutes while rotating. Samples were centrifuged at 4°C until reaching 500 rpm and elutants were collected. These elutants contain our protein. Samples were again incubated with glutathione-agarose beads and eluted with Glut-Elute twice more to ensure a high recovery of GST protein. GST-fusion proteins were run on SDS-PAGE and stained to determine size and amount of recovered protein.

Smad2 Immunocytochemistry

NBFL cells were plated onto collegen coated plates, and stimulated with activin A (100ng/ml) for 60 minutes prior to being fixed in Zamboni's fix (4% paraformaldehyde, 15% picric acid in 0.1M PO4, pH 7.2) for 20 minute. These cultures, together with untreated sister cultures, were then preincubated in a blocking buffer containing 10% horse serum, 0.5M NaCl, 0.1M PO4, pH 7.2, 0.1% triton-X-100 and .01% Na azide. The

plates where then reacted overnight with an anti-smad 2 goat antiserum (Santa Cruz Biotechnology) diluted 1:400 in the blocking buffer. Binding of the primary antibodies was made visible using a rabbit anti-goat antiserum conjugated with Cy3 (Jackson Immuno Labs) diluted 1:800 in the blocking serum for 1 hour. Controls included substitution of primary antisera with normal serum at an approximately equivalent immunoglobulin concentration.

Table 1. Sequences of oligonucleotides used in EMSA

oligonucleotide	5'	3'
M3G2	GC CGG AGC TTA ATC ATA TTT T	
AP-1	GGC TTG ATG AGT CAG CCG GAA	
P3	GGG GAT TTC CTG GAA TTA AG	
SBE	GGG AGT ATG TCT AGA CTG ACA ATG TA	
P15	GAT CCA AAT GTC TGG AAA TTT GTT TCC CAG TTG A	
MP15	GAT CCA AAT GTC T**AT** **CTG** TTT GTT TCC CAG TTG A	
P17	GAT CCA TTT CCA GAC ATT TTG AAA CTT AAT TCA TTT A	
MP17	GAT CCA TTT CCA **TCG** ATT TTG AAA CTT AAT TCA TTT A	
P18	GAT CCA TTT AAT TTT TCT GGT AAC TGG ATT AGA AAA TA	
P11	GGG TGG ATC AGT CTG ACT TTG AAC G	
MP11	GGG TGG ATC A**TA** **G**TG ACT TTG AAC G	

*nucleotides in bold indicate mutations

Results

To determine whether or not TGF-β, alone or in combination with CNTF, regulates VIP gene expression in NBFL cells.

Members of the transforming growth factor-beta (TGF-β) and the gp130-mediated cytokines are known modulators of neuropeptide expression. To understand further the mechanism by which these external signals regulate gene expression, we looked for a model in which both cytokines could be studied. Ciliary Neurotrophic Factor (CNTF), a member of the gp130-cytokine family, has previously been shown to regulate the expression of a number of neuropeptide genes, including vasoactive intestinal peptide (VIP) (Symes et al., 1993).

In an attempt to determine whether TGF-β alone or in combination with CNTF induced VIP expression, NBFL neuroblastoma cells were treated with CNTF (25 ng/ml), TGF-β (1 ng/ml or 10 ng/ml), or a combination of the two cytokines for 48 hours. RNA was isolated and northern blot analysis performed to detect expression of VIP mRNA (Figure 3). To normalize for loading differences, the blot was also probed for the ubiquitously expressed protein cyclophillin. Compared to untreated controls, CNTF induced expression of VIP mRNA approximately 56 fold. TGF-β also induced VIP mRNA expression (3-12 fold) in NBFL cells when compared to untreated cells. Interestingly, a robust increase in VIP mRNA expression (147-257 fold) was observed when both cytokines were used. This effect was more than additive, signifying a synergistic interaction between these two signaling pathways.

Previous characterization of the VIP promoter revealed a region that was necessary and sufficient for gp130 cytokine-mediated regulation of VIP transcription. This region of the VIP promotor, a 180 bp sequence, termed the Cytokine Response Element (CyRE) (Symes et al., 1994) (Figure 4). At least two signal transduction pathways are utilized by gp130-mediated cytokines: the JAK-STAT and the AP-1 pathway. A STAT binding site was found in the 5' region of the VIP CyRE (Symes et al., 1994) along with an AP-1 binding site in the 3' region (Symes et al., 1997). Both are important in CNTF-mediated signaling through this element.

To characterize the nature of TGF-β signaling and its synergy with CNTF, NBFL cells were transiently transfected with luciferase reporter plasmids containing the entire 180 bp CyRE upstream of a minimal (90 bp) RSV promotor and a luciferase gene. This construct is referred to as Cy1Luc. Cells were treated with CNTF (25 ng/ml), TGF-β (2.5 ng/ml) or both cytokines for 36 hours and the relative levels of luciferase activity measured (Figure 5). The level of luciferase activity is believed to be proportional to the amount of transcription directed by the promoter elements (Gorman, et al., 1982; de Wet, et al., 1987; Wood, 1990; Alam and Cook, 1990). CNTF induces CyRE-mediated transcription approximately 40 fold over that in untreated transfected cells. Consistent with the induction of VIP mRNA, TGF-β induced Cy1Luc activity 13 fold compared to 40 fold for CNTF. Both northern blotting and transfection studies suggest that the CyRE is necessary and sufficient to mediate VIP regulation by two completely distinct signaling pathways. Treatment with both cytokines induced an approximately 200 fold increase in luciferase activity of Cy1Luc over untreated controls. This lends more evidence to the hypothesis of a synergistic interaction between these two pathways.

Figure 3. TGF-β individually and synergistically with CNTF upregulates VIP mRNA levels

NBFL cells were treated with CNTF (25 ng/ml), TGF-β (1 ng/ml or 10 ng/ml), or a combination of the two for 48 hours. RNA was isolated and northern blot analysis performed. CNTF induces expression of VIP mRNA approximately 56 fold whereas TGF-β only induces between 3-12 fold. Treatment with CNTF and TGF-β leads to a synergistic induction of VIP mRNA (147-257 fold).

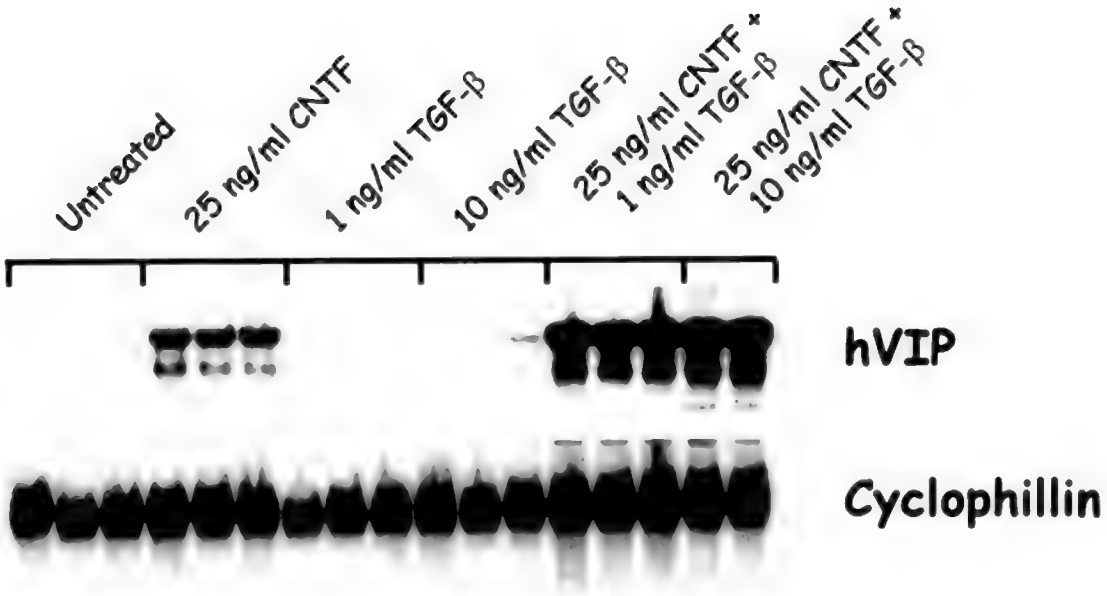

Untreated
25 ng/ml CNTF
1 ng/ml TGF-β
10 ng/ml TGF-β
25 ng/ml CNTF *
1 ng/ml TGF-β
25 ng/ml CNTF *
10 ng/ml TGF-β
hVIP
Cyclophillin

Figure 4. CyRE Sequence and corresponding binding sites

The Cytokine Response Element is a 180 bp region of the VIP promoter that contains multiple transcription factor binding sites necessary for gp130-mediated cytokine signaling. A region of the 5' end contains a STAT transcription factor binding site corresponding to the oligo labeled P3. A region toward the 3' end contains a binding site for an AP-1 complex. The corresponding oligo is labeled P2. Other regions investigated in this body of work are also identified. The presence of multiple sites within the CyRE being utilized by different external signals implies a sophisticated and intricate mechanism of regulation.

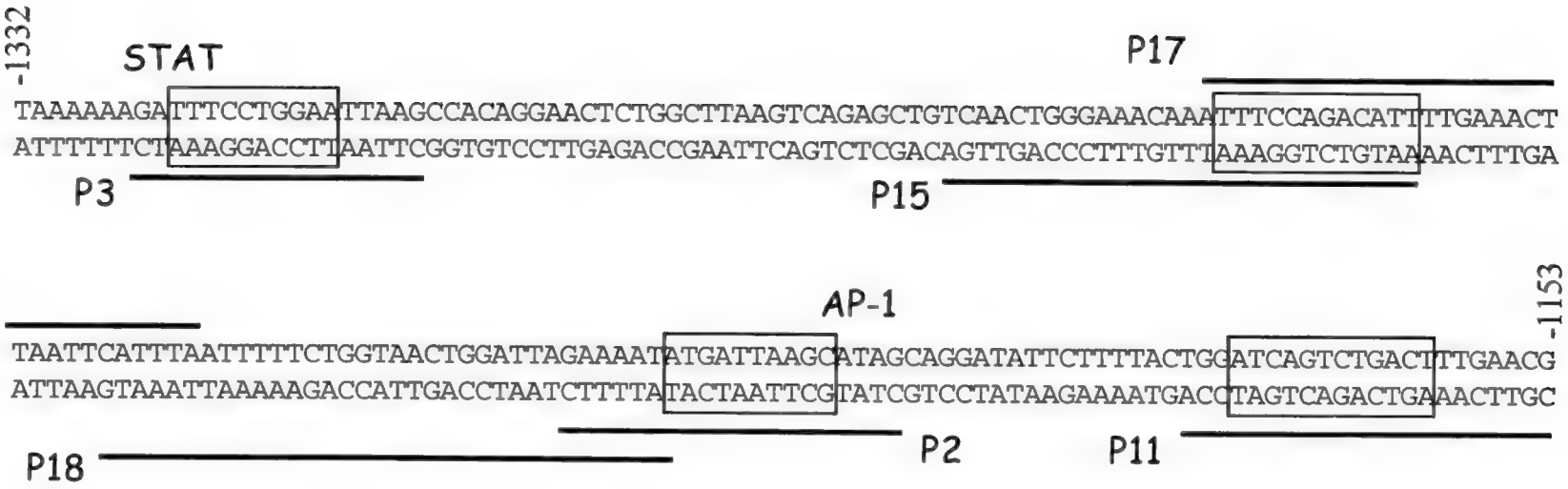
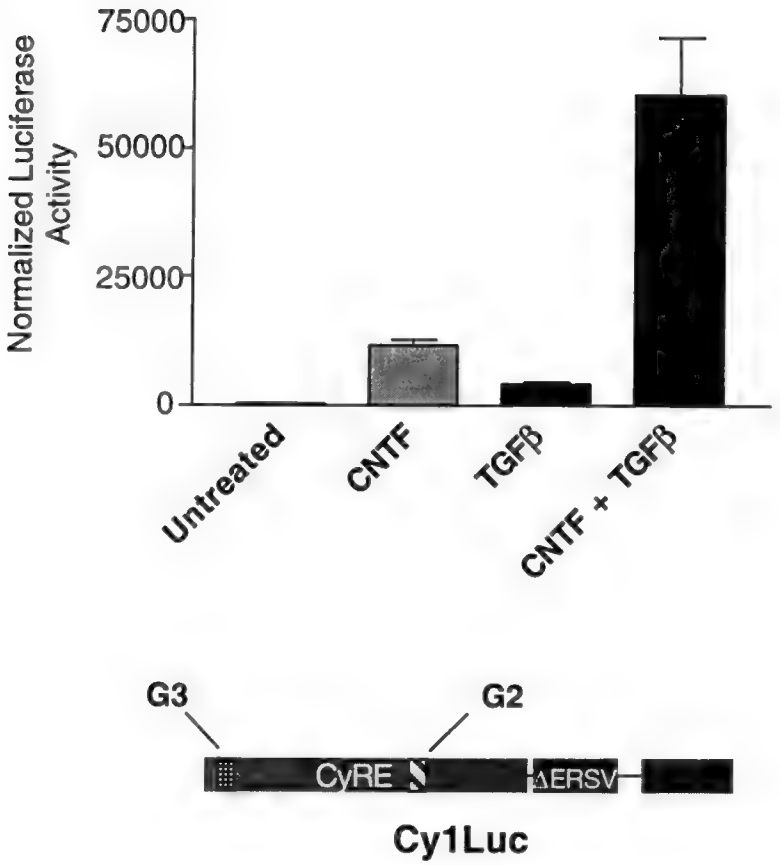

-1332
STAT
P17
TAAAAAAGATTTCCTGGAATTAAGCCACAGGAACTCTGGCTTAAGTCAGAGCTGTCAACTGGGAAACAAATTTCCAGACATTTTGAAACT
ATTTTTTCTAAAGGACCTTAATTCGGTGTCCTTGAGACCGAATTCAGTCTCGACAGTTGACCCTTTGTTTAAAGGTCTGTAAAACTTTGA
P3
P15
AP-1
-1153
TAATTCATTTAATTTTTCTGGTAACTGGATTAGAAAATATGATTAAGCATAGCAGGATATTCTTTTACTGGATCAGTCTGACTTTGAACG
ATTAAGTAAATTAAAAAGACCATTGACCTAATCTTTTATACTAATTCGTATCGTCCTATAAGAAAATGACCTAGTCAGACTGAAACTTGC
P18
P2
P11

Figure 5. The VIP CyRE is necessary and sufficient for CNTF and TGF-β mediated signal transduction

NBFL cells were transiently transfected with the Cy1Luc reporter plasmid containing a luciferase gene driven by the 180 bp VIP CyRE upstream of a basal RSV promoter. Cells were treated for 36 hours with CNTF (25 ng/ml), TGF-β (2.5 ng/ml), or both cytokines. CNTF induced luciferase activity of Cy1Luc approximately 40 fold whereas TGF-b induced Cy1Luc approximately 13 fold. Treatment with both cytokines synergistically induced Cy1Luc approximately 200 fold. Luciferase values were normalized to the level of β-galactosidase expression.


Normalized Luciferase Activity
75000
50000
25000
0
Untreated
CNTF
TGFβ
CNTF + TGFβ
G3
G2
CyRE
ΔERSV
Cy1Luc

To determine whether or not the regulation of VIP by CNTF and TGF-β is through separate, independent signaling pathways

To understand further the interaction between these two signaling pathways, experiments were performed to elucidate the signal transduction pathways for each cytokine and the point of convergence between the two. Activation of STAT and AP-1 transcriptional complexes is known to mediate CNTF signaling (Symes, et al., 1997). Using a luciferase reporter gene construct under the control of multimerized binding sites for either AP-1 or STATs, the effect of TGF-β and CNTF signaling on these transcription factors was ascertained. NBFL cells were transfected with constructs containing 3 multimerized STAT (3xG3) or AP-1 (3xG2) binding sites from the CyRE upstream of a minimal (90 bp) RSV promoter and a luciferase gene. Cells were treated with CNTF (25 ng/ml), TGF-β (2.5 ng/ml), or a combination of both for 36 hours and assayed for levels of luciferase activity (Figure 6). Compared to untreated controls, CNTF did not significantly induce expression through the AP-1 driven construct but did induce a 2 fold induction in transcription through the STAT-driven construct. TGF-β reduced luciferase activity of 3xG3 and 3xG2 approximately 50% and 70%, respectively, below untreated transfected cells. TGF-β also reduced CNTF-mediated induction of 3xG3 and 3xG2 approximately 30% and 49%, respectively. These data indicate that TGF-β suppresses transcription through these constructs and thus does not stimulate expression of VIP through either STAT or AP-1 binding sites alone.

To look for STAT or AP-1 activation at the level of DNA binding, electrophoretic mobility shift assays (EMSAs) were performed on nuclear extracts of NBFL cells treated

with cytokines for various amounts of time. Cells were treated for 15 minutes, 1 hour, 3 hours, and 6 hours with CNTF (25 ng/ml), TGF-β (5 ng/ml), or a combination of the two. Radioactive probes of the CyRE-STAT binding site, P3, and a mutated CyRE-AP-1 binding site, M3G2, were used. The mutation in M3G2 enhances the binding of the AP-1 complex (Symes, et al., 1997). In panel A (Figure 7), nuclear extracts prepared from NBFL cells treated at various time points with these cytokines were incubated with the P3 probe. Binding of proteins to the P3 probe is an indication of STAT activation. As was previously seen, CNTF induces binding of STAT proteins within 15 minutes of treatment. TGF-β treatment neither induced STAT proteins to bind nor altered CNTF-mediated STAT binding. In panel B (Figure 7), the mutated AP-1 probe, M3G2 was incubated with this same set of nuclear extracts. As previously seen, CNTF induces binding of AP-1 complexes to a site in the CyRE within 1 hour. Treatment of TGF-β did not induce AP-1 binding to M3G2. TGF-β appears to reduce CNTF-activated AP-1 binding to the M3G2 probe (panel B, lanes 11-15). This most likely is the result of unequal gel loading as TGF-β was observed in replicate experiments to not alter CNTF-mediated AP-1 binding (data not shown). In panel C (Figure 7), a consensus AP-1 site was used as a probe and incubated with the same set of NBFL nuclear extracts. The results are similar to those seen in panel B. TGF-β neither induced AP-1 binding nor altered CNTF-mediated AP-1 binding. Taken together, both EMSA and transfection data suggest separate and distinct signaling pathways are used by CNTF and TGF-β to induce VIP gene transcription through the VIP CyRE in NBFL cells.

Figure 6. Regulation of Cy1Luc by TGF-β is not due to STAT or AP-1 signal transduction alone

NBFL cells were transiently transfected with reporter plasmids containing a luciferase gene controlled by multimerized STAT (3xG3) and AP-1 (3xG2) sites from the CyRE. Cells were treated for 36 hours with CNTF (25 ng/ml), TGF-β (2.5 ng/ml), or both

NBFL cells were transiently transfected with reporter plasmids containing a luciferase

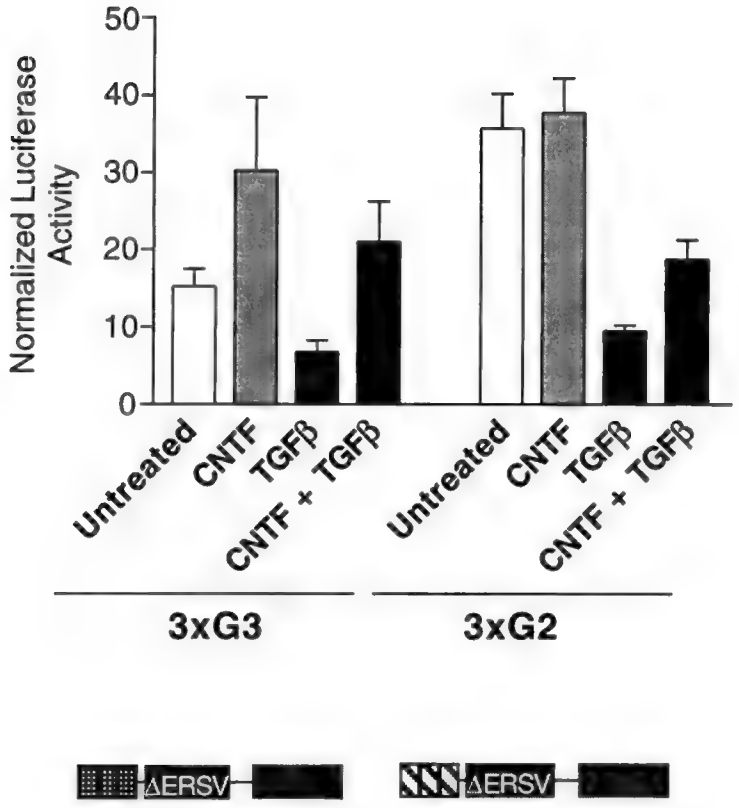
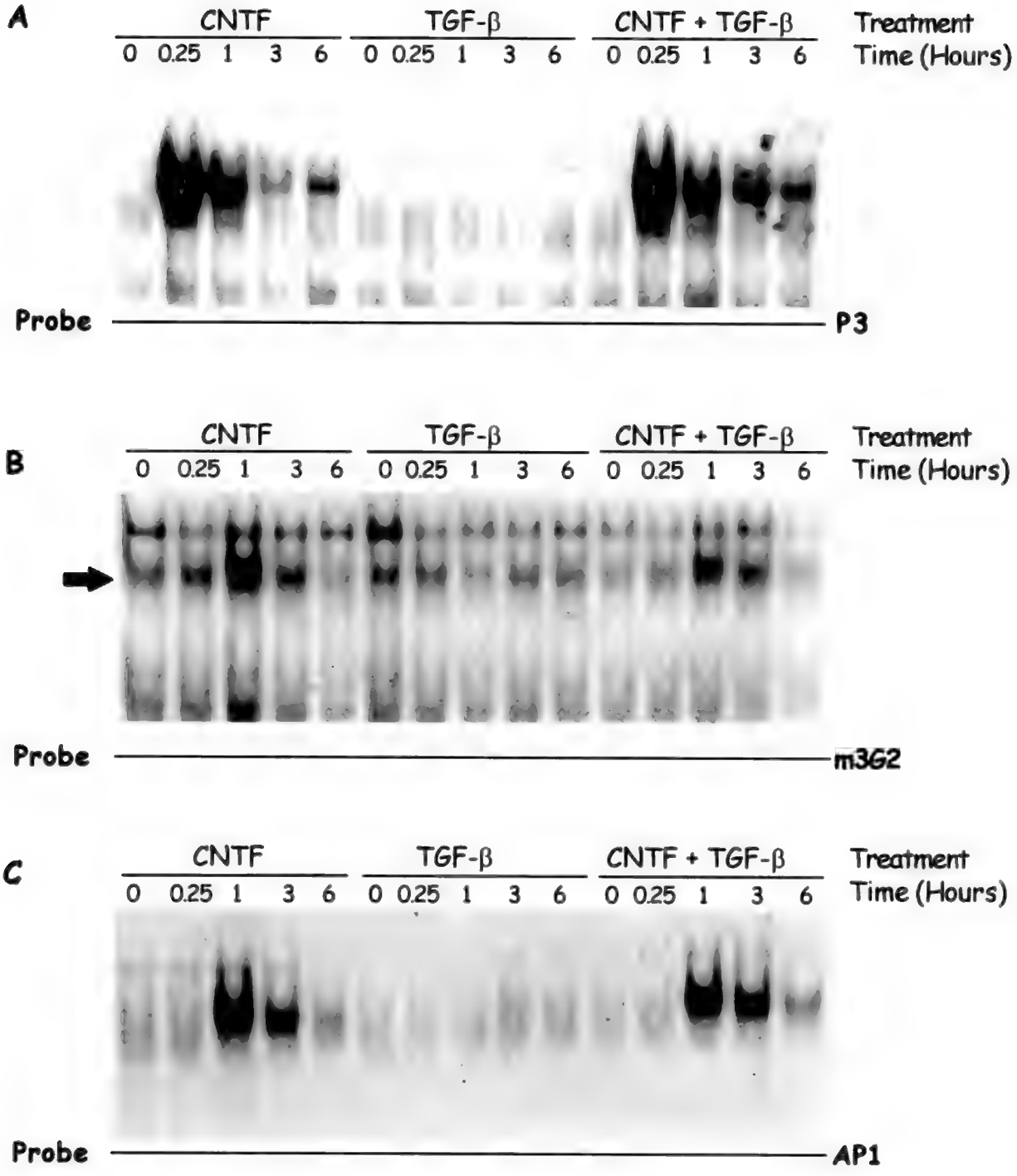

Normalized Luciferase Activity
50
40
30
20
10
0
Untreated
CNTF
TGFβ
CNTF + TGFβ
Untreated
CNTF
TGFβ
CNTF + TGFβ
3xG3
3xG2
ΔERSV
ΔERSV

Figure 7. CNTF-mediated signaling induces binding of STAT and AP-1 transcription factors to the CyRE

Induction of STAT and AP-1 binding was assessed by EMSA using probes corresponding to known sites within the CyRE, P3 and P2 respectively. NBFL cells were treated with CNTF (25 ng/ml) and/or TGF-β (2.5 ng/ml) for various amounts of time. Nuclear extracts were prepared and binding to STAT and AP-1 probes assessed. CNTF induces STAT binding after 15 minutes of treatment, which isn't seen or altered by TGF-β treatment. M3G2 is a probe similar to P2 with a mutation to intensify the binding of AP-1 proteins. Binding to a consensus AP-1 site was also assessed by use of a canonical AP-1 probe. Both probes show CNTF induction of AP-1 binding within an hour. This induction is not seen with TGF-β. Although TGF-β appears to reduced CNTF-mediated AP-1 binding in Panel B, replicate experiments indicate that TGF-β treatment does not alter the induction of AP-1 binding by CNTF.


A
CNTF
TGF-β
CNTF + TGF-β
Treatment
Time (Hours)
0 0.25 1 3 6 0 0.25 1 3 6 0 0.25 1 3 6
Probe
P3
B
CNTF
TGF-β
CNTF + TGF-β
Treatment
Time (Hours)
0 0.25 1 3 6 0 0.25 1 3 6 0 0.25 1 3 6
Probe
m3G2
C
CNTF
TGF-β
CNTF + TGF-β
Treatment
Time (Hours)
0 0.25 1 3 6 0 0.25 1 3 6 0 0.25 1 3 6
Probe
AP1

To determine whether Smad proteins are present in NBFL cells and may mediate CNTF signals in addition to those of TGF-β

Smad proteins are known mediators of TGF-β signaling. We sought to identify smad activation and translocation in NBFL cells. Immunohistochemistry was performed to ascertain whether NBFL cells express smad transcription factors (Figure 8). NBFL cells were treated for 1 hour with Activin (100 ng/ml), a TGF-β superfamily member. Cells were then fixed and stained with an antisera that recognizes an epitope common to both Smad 2 and Smad 3. In panel A (Figure 8), the presence and localization of smad immunoreactivity appears to be ubiquitous and diffuse in untreated cells. Smad localization is predominately cytoplasmic. Activin treated cells exhibited nuclear smad immunoreactivity (panel B, Figure 8). Our data is consistent with the model that smad proteins are activated and translocate to the nucleus after TGF-β/Activin receptor-mediated activation of the signaling pathway.

Western blots were performed to characterize further the time course of Smad activation and translocation. Nuclear extracts were made from NBFL cells treated with TGF-β (5 ng/ml) for 15 minutes, 1 hour and 3 hours. Cos7 cells were transfected with expression plasmids for Smad 2 and Smad 3 and cellular lysates were prepared. These lysates served as a control for positive identification of Smad 2 or Smad 3 in NBFL cells. TGF-β treated nuclear extracts were exposed to denaturing PAGE conditions and western analysis performed using the same Smad2/3 recognizing antibody. The antibody recognized both positive control lysates and Smad 2 and 3 could be distinguished easily based on varying molecular weights. Smad nuclear translocation was detected after 15

minutes of TGF-β treatment (Figure 9). Identification of Smad subtype was difficult because the molecular weight of the protein detected fell between both positive controls. Thus TGF-β induced activation and translocation of Smad transcription factors in NBFL cells but composition of this translocating Smad complex is unclear.

Another way to look at TGF-β activation of smad pathways is through endogenous smad-mediated transcription. In a representative experiment, NBFL cells were transfected with luciferase reporter plasmids under the control of multimerized Smad Binding Elements (SBE-Luc). Cells were treated with CNTF (25 ng/ml), TGF-β (2.5 ng/ml), and both cytokines for 36 hours and luciferase activity was assayed. CNTF treated cells resulted in no induction of luciferase activity beyond that seen in untreated cells. TGF-β (2.5 ng/ml) induced luciferase activity of SBE-Luc 12.5 fold (Figure 10). Treatment with both cytokines resulted in a 13.5 fold increase in luciferase activity of SBE-Luc. Thus TGF-β appears to activate smad-mediated transcription through SBE-Luc whereas CNTF does not, suggesting CNTF is not an activator of smad proteins.

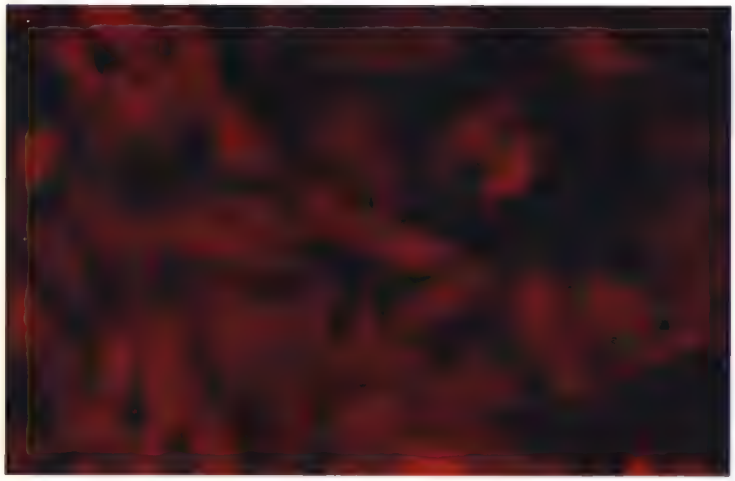
To determine whether CNTF in addition to TGF-β would activate smad-mediated transcription, we decided to utilize chimeric Gal4-smad fusion proteins. These experiments enabled us to examine potential CNTF activation of smad transcription independent of smad binding to DNA. In a representative experiment, chimeric Gal4-smad proteins were transfected into NBFL cells and cytokine stimulation of their activation studied. Cells were also transfected with a luciferase reporter gene under the control of multimerized Gal4-DNA binding sites (pGE5-Luc). Cytokine stimulated phosphorylation and activation of chimeric smad proteins should result in chimeric Gal4-smad binding to Gal4 sites, thus activating transcription through the pGE5-Luc construct.

Cells were treated with CNTF (25 ng/ml), TGF-β (2.5 ng/ml), or a combination of the two cytokines for 36 hours and luciferase activity was assayed (Figure 11). CNTF treatment (25 ng/ml) induced 1.5 and 2.5 fold increases in luciferase activity of pGE5-Luc over untreated cells in the presence of Gal4-Smad 2 and Gal4-Smad4, respectively. CNTF did not stimulate transcription in the presence of Gal4-Smad3. In contrast, TGF-β treatment (2.5 ng/ml) did stimulate increased luciferase activity (3 fold) of pGE5-Luc in the presence of Gal4-Smad3. TGF-β also induced 3 and 3.5 fold increases in luciferase activity beyond untreated controls in cells containing Gal4-Smad2 and Gal4-Smad4, respectively. In the presence of Gal4-Smad3, treatment with both cytokines stimulated a 4 fold increase in luciferase activity. When treated with both cytokines, Gal4-Smad2 and Gal4-Smad4 did not exhibit increased luciferase activity beyond that seen with TGF-β treatment alone. Our data suggests that TGF-β treatment equally activates Smad 2 and Smad 3 in NBFL cells. CNTF was also able to activate Smad 2. Further increases in transcription with both cytokines were seen only with Smad 3. Thus our data suggests we have functional, endogenous smad proteins present in NBFL cells that are responsive to TGF-β and possibly CNTF.

Figure 8. Smad 2/3 translocation into the Nucleus after Activin treatment.

NBFL cells were treated with Activin (100 ng/ml) and stained with an antibody that recognizes epitopes for both Smad 2 and Smad 3. Panel A shows untreated cells. Staining for smad proteins in these cells is diffuse and cytoplasmic. Panel B shows Activin-treated cells. Staining in these cells is localized primarily in the nucleus. Thus Activin treatment may be stimulating smad nuclear translocation, which would be consistent with current models of TGF-β signaling.

A



B

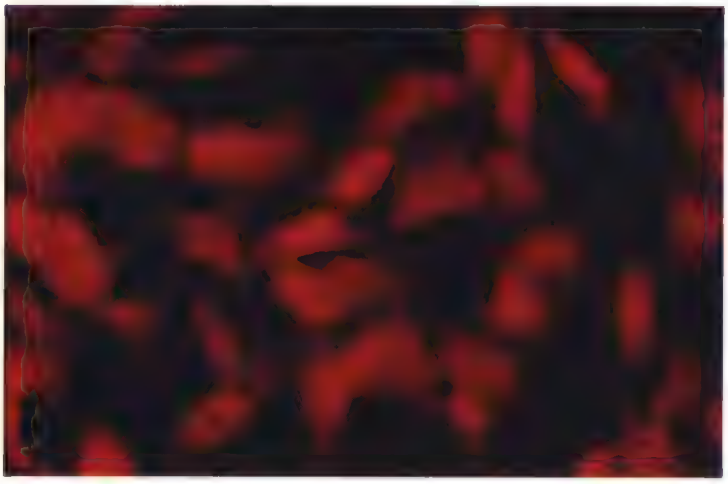


Figure 9. Smads translocate to the nucleus within 15 minutes of treatment.

Nuclear extracts were prepared from NBFL cells treated with TGF-β (5 ng/ml) and harvested at various time points. Cos7 cells were transfected with expression plasmids for Smad 2 and Smad 3 and lysates prepared to act as positive controls. Western blotting with an antibody that recognizes both Smad 2 and Smad 3 (Upstate Biotechnology, Inc.) revealed the nuclear accumulation of a protein intermediate in size to Smad 2 and Smad 3 after 15 minutes of TGF-β treatment.

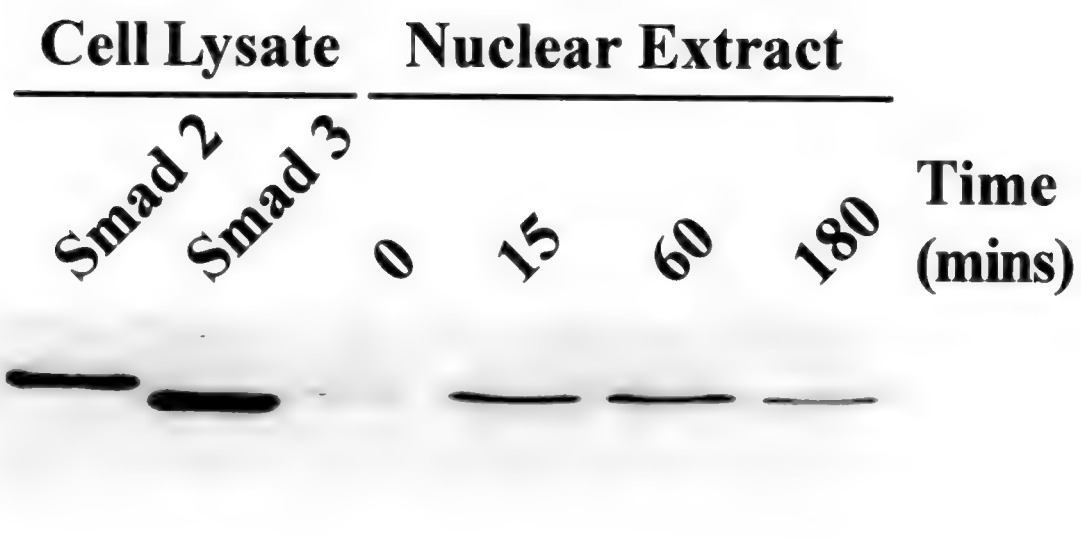

Cell Lysate
Nuclear Extract
Smad 2
Smad 3
0
15
60
180
Time (mins)

Figure 10. TGF-β but not CNTF is able to stimulate endogenous smad mediated transcription

NBFL cells were transfected with luciferase reporter plasmids under the control of multimerized smad binding elements (SBE-Luc). Cells were treated with CNTF (25ng/ml), TGF-β (2.5 ng/ml), and both cytokines for 36 hours and luciferase activity assayed. While TGF-β stimulated luciferase levels 12.5 fold, CNTF did not induce transcription driven by this construct. These data suggest that CNTF is not able to activate smad-mediated transcription in NBFL cells.

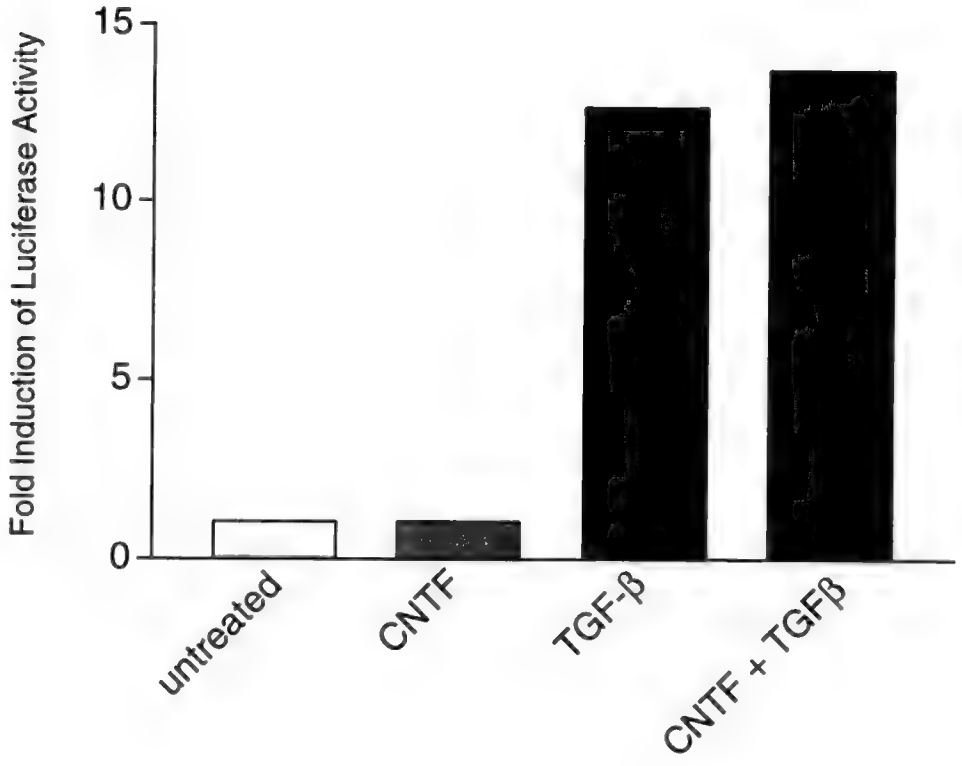
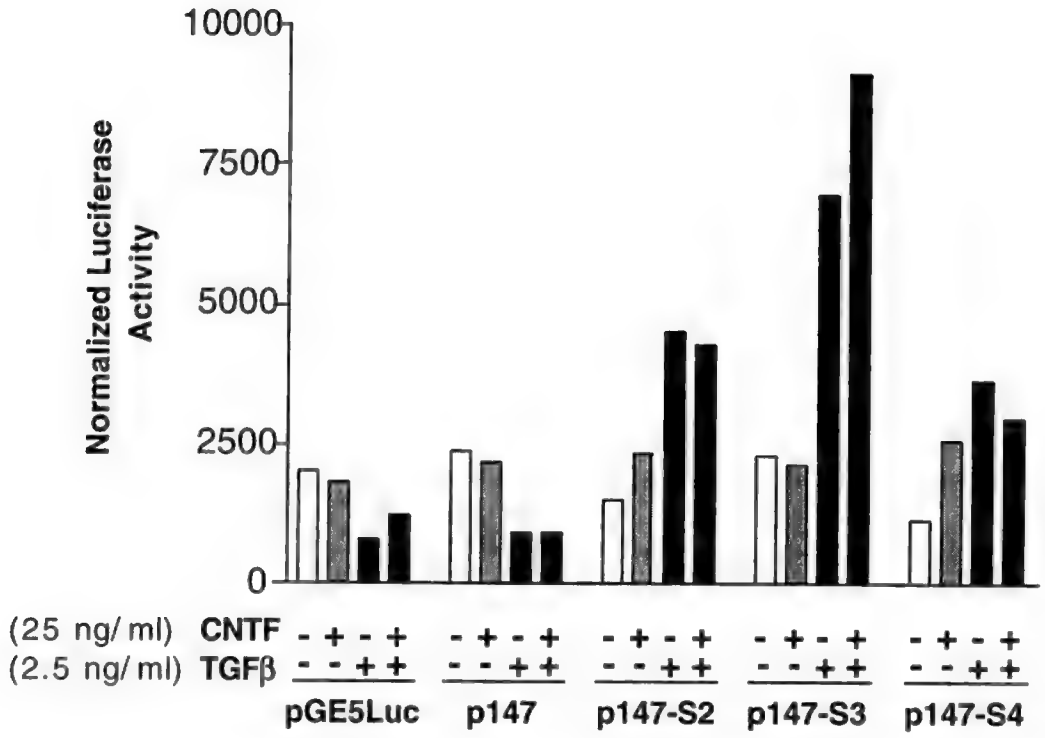
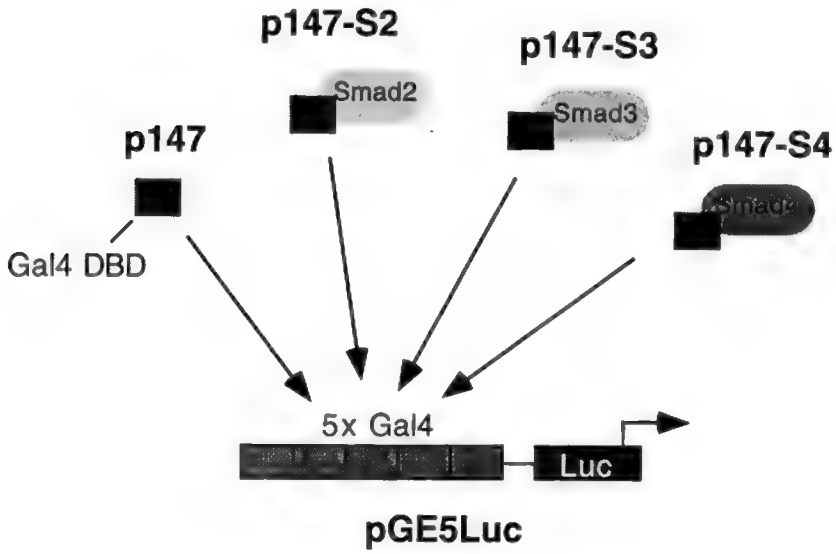

Fold Induction of Luciferase Activity
15
10
5
0
untreated
CNTF
TGF-β
CNTF + TGFβ

Figure 11. TGF-β activates chimeric Smad 2 and Smad 3 proteins in NBFL cells

NBFL cells were transfected with expression plasmids for proteins that contained smad transactivation domains fused to a Gal4 DNA-binding domain and for proteins that contained only Gal4 DNA-binding domains. Cells were also transfected with a luciferase reporter plasmid under the control of 5 copies of a Gal4 binding site (pGE5-Luc). Cells were treated with CNTF (25 ng/ml), TGF-β (2.5 ng/ml), or both cytokines for 36 hours and luciferase and β-galactosidase activity assayed. TGF-β treatment in the presence of Smad 2 and Smad 3 resulted in increased luciferase activity above basal levels. CNTF was also observed to activate Smad 2 slightly. Thus TGF-β can activate Smad 2 and Smad 3 in NBFL cells.


10000
7500
5000
2500
0
Normalized Luciferase Activity
(25 ng/ml) CNTF - + - + - + - + - + - + - + - + - + - +
(2.5 ng/ml) TGFβ - - + + - - + + - - + + - - + + - - + +
pGE5Luc
p147
p147-S2
p147-S3
p147-S4


p147-S2
Smad2
p147-S3
Smad3
p147
p147-S4
Gal4 DBD
5x Gal4
Luc
pGE5Luc

To determine whether Smad proteins can bind to and activate transcription through the VIP CyRE

Since TGF-β induces VIP gene expression through the VIP CyRE in NBFL cells, we wanted to determine which regions of the CyRE were necessary for this induction. To identify such regions, several CyLuc constructs with sequential 5' and 3' deletions were transfected into NBFL cells and treated with TGF-β (2.5 ng/ml). Cells were treated for approximately 36 hours and assayed for luciferase activity. Deletion of the first 30 bps of the 5' end of the CyRE (Cy7Luc) decreased TGF-β responsiveness to Cy1Luc by approximately 54%. Deletions that involved more than 30 bps resulted in approximately 80-90% reduction in TGF-β responsiveness on average. Although all deletional constructs saw a reduction in TGF-β responsivness, a single deletion of the most 3' 28 bps of the CyRE (Cy2Luc) abrogated approximately 77% of TGF-β responsiveness to Cy1Luc (Figure 12). Interestingly, the basal levels of luciferase activity directed by Cy2Luc are much greater than those of Cy1Luc. This phenomena was also observed with CNTF in similar experiments (data not shown) and suggest that a repressor may be binding to the 3' CyRE. All CyRE-deletion constructs saw a marked deletion in TGF-β responsiveness, suggesting multiple regions may contribute to TGF-β induction of CyRE transcription.

Because of the substantial loss of TGF-β induced activity with Cy2Luc, we decided to characterize further the 3' region of the CyRE. A series of mutants was created with the introduction of sequential 3 bp mutations along the first 30 bps of the 3' end of the CyRE (Jones, et al., 2000). These mutated sequences were placed upstream of

a luciferase gene and a minimal RSV promoter. These mutational constructs, mS1-10, were transfected into NBFL cells and treated with TGF-β (2.5 ng/ml) for 36 hours. Luciferase activity was assayed and normalized to β-galactosidase activity (Figure 13). Many of the mutants resulted in decreased luciferase activity but the largest decrease was seen with mS4 and mS5, which corresponds to the region coded GTCTGA. Interestingly this region TCA GTC TGA has sequence similarity to a Smad binding element, CA GAC AGA. Thus the 3 bp mutation GTC to TAG abrogated TGF-β mediated transcription, possibly due to interrupted smad binding.

The possible existence of a smad binding site suggested that smads might directly bind to DNA in the CyRE. Binding of native Smad3/Smad4 complexes to the CyRE proved to be technically difficult as we were unable to find an inducible binding complex with EMSA (data not shown). Thus, to investigate the direct binding of smad proteins to DNA, purified Smad proteins were produced by expression of Smads fused to a glutathione-S-transferase tag. A full length Smad 4 (GST-Smad4) and a truncated Smad 3 (GST-ΔcSmad3) were expressed in E. coli. Bacterially expressed proteins were purified by binding to glutathione beads and purified proteins were used in EMSAs. Oligonucleotides corresponding to multiple regions of the CyRE were synthesized, annealed, and radiolabeled as probes.

Using a known smad binding element (SBE) as a positive control, two regions were identified that bound GST-ΔcSmad 3. In lanes 1-3 (Figure 14), GST-ΔcSmad3 binds to the SBE specifically when compared to purified GST alone, showing that the bacterially expressed truncated Smad 3 proteins are binding to specific smad sites. In lanes 4-9 (Figure 14), the same specific binding is observed with probes for the region

P15/P17. These sequences overlap in a region that has sequence similarity to a smad binding element. When an adjacent sequence (P18) is used as a probe (lanes 10-12), no specific binding is observed. The second site, P11, corresponds to the possible 3' CyRE smad binding site. GST-ΔcSmad3 also binds to P11 (lanes 10-12). Binding of GST-ΔcSmad3 to P11 is weaker than to P17, as seen by decreased band intensity.

To demonstrate that both purified GST-ΔcSmad3 and GST-Smad4 are able to bind DNA, the proteins were incubated with the positive control sequence, SBE. In the second panel on the bottom (Figure 14), both GST-ΔcSmad 3 and GST-Smad 4 bind to the SBE. Because Smad 4 binds with a relatively weaker affinity that the truncated Smad 3, a separate gel was run to determine Smad 4 binding to these two putative sites. In the third panel on the bottom (Figure 14), GST-Smad 4 indeed binds weakly to both sites. Because of the dramatic difference in binding affinities, GST-ΔcSmad 3 was used for the remainder of the EMSA experiments.

If these sites are binding Smad proteins specifically, then mutations within those sites should interrupt protein-DNA interaction. Mutated oligonucleotides were made in the regions of P15, P17, and P11 and used as cold competitors in EMSA. In panel A of Figure 15, each probe is in competition with 100X cold oligonucleotides of itself, the other putative smad site, the positive control sequence (SBE), and a non-smad binding sequence (AP-1). P15 is completely competed by a cold 100X excess of itself and the SBE while moderately competed by the other putative site, P11. The same pattern was seen with P17, with SBE and P11 moderately competing. In contrast, P11 binding was competed by itself, P17, and the SBE. AP-1 does not compete for smad binding. These

data show that there are two smad sites within the VIP CyRE that can bind a truncated form of Smad 3.

To specifically determine whether GST-ΔcSmad 3 was binding to the DNA through the site we had identified as a potential SBE, we synthesized mutated versions of P15, P17, and P11 with 3 bp mutations in the smad site. These mutated oligonucleotides were used to compete for GST-ΔcSmad3 binding to the wild type probes (Figure 15, Panel B). If the site is crucial for smad binding, then mutated sites should not be able to bind GST-ΔcSmad3 and therefore not compete for GST-ΔcSmad3 protein binding. Both radiolabeled P17 and P11 were competed with 100 fold molar excess of unlabelled P15, P17, and P11. Mutant oligonucleotides were unable to compete for GST-ΔcSmad3 binding, suggesting that the specific mutations we introduced removed the Smad 3 binding site.

Previous experiments have shown TGF-β to induce Cy1Luc activity approximately 13 fold (Figure 5). If these smad binding sites are critical to TGF-β mediated induction of Cy1Luc, then mutations of these should reduce or eliminate TGF-β induction. We therefore introduced mutations in these sites into Cy1Luc. Mutations were made in the AP-1 site (Cy1mP2Luc), the first smad binding site (Cy1mP15Luc and Cy1mP17Luc), and the second smad binding site (Cy1mP11Luc). Cy1Luc and the series of CyLuc mutants were transfected into NBFL cells and treated with TGF-β (2.5 ng/ml) for 36 hours. Luciferase activity was assayed and normalized against β-galactosidase levels (Figure 16). As previously seen, TGF-β induces Cy1Luc directed luciferase activity approximately 11 fold. When mutations were introduced into either smad binding site (Cy1mP15Luc, Cy1mP17Luc, or Cy1mP11Luc), a marked diminution of

response to TGF-β was seen. Mutations in P11 and P15/P17 smad sites saw reductions of TGF-β responsiveness to Cy1Luc by 86% and 90%, respectively. The greatest reduction was seen with the mutation in the P15/P17 region. Interestingly, when mutations were introduced into the AP-1 site (Cy1mP2Luc), the TGF-β responsiveness dropped by 60%. Although TGF-β did not alter AP-1 binding in this cell line, these data suggest that there still might be some protein-protein interactions important to the TGF-b response that are disrupted by this mutation. These data indicate that both CyRE Smad sites are functional and contribute to TGF-β responsiveness of CyRE transcription.

Figure 12. A region in the 3' end of the VIP CyRE is involved in TGF-β mediated induction

NBFL cells were transiently transfected with 3' and 5' deletional constructs of the Cy1Luc reporter and treated with (+) or without (-) TGF-β (2.5 ng/ml) for 36 hours. Each deletion of the 3' or 5' end involved approximately 30 bps. All constructs resulted in reduced TGF-β responsiveness; the most prominent reduction by a single ~30 bp deletion was seen in deletion of the most 3' 28 bps of the CyRE. Luciferase values were normalized to the level of β-galactosidase expression.

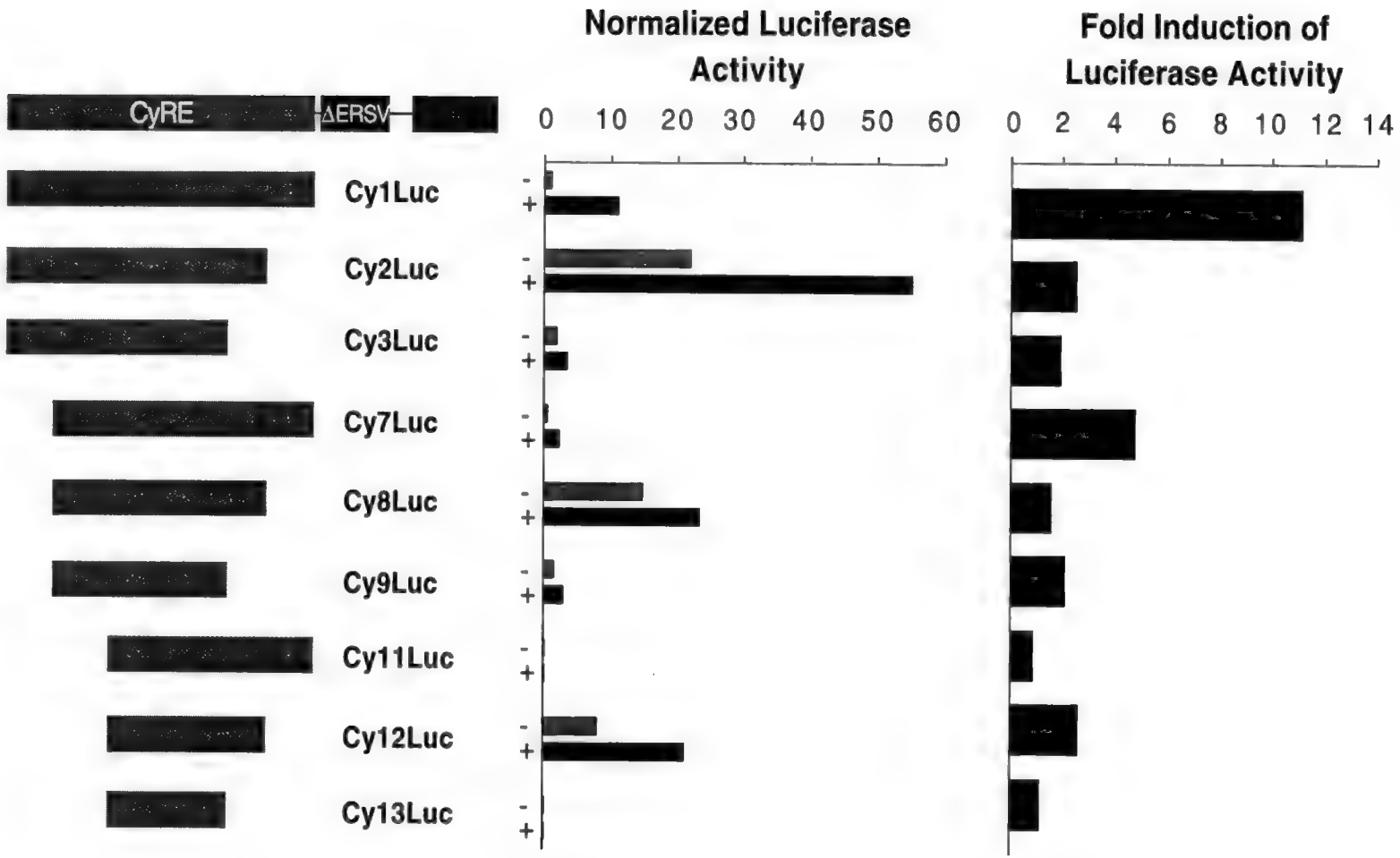

Normalized Luciferase Activity
Fold Induction of Luciferase Activity
CyRE
ΔERSV
0 10 20 30 40 50 60
0 2 4 6 8 10 12 14
Cy1Luc
Cy2Luc
Cy3Luc
Cy7Luc
Cy8Luc
Cy9Luc
Cy11Luc
Cy12Luc
Cy13Luc
-
+

Figure 13. Mutational analysis of the 3' region identifies a TGF-β responsive element

NBFL cells were transient transfected with a series of mutational constructs based on the Cy1Luc reporter and treated with (+) or without (-) TGF-β (2.5 ng/ml) for 36 hours. Sequential 3 bp mutations introduced into the 3' end of the Cy1Luc reporter plasmid allowed identification of regions necessary for TGF-β mediated induction. Mutations labeled mS4 and mS5 demonstrated the most pronounced reduction in TGF-β responsiveness. The region mutated in these sites (GTCTGAC) is homologous to a consensus smad binding element (CAGAC). Luciferase values were normalized to the level of β-galactosidase expression.

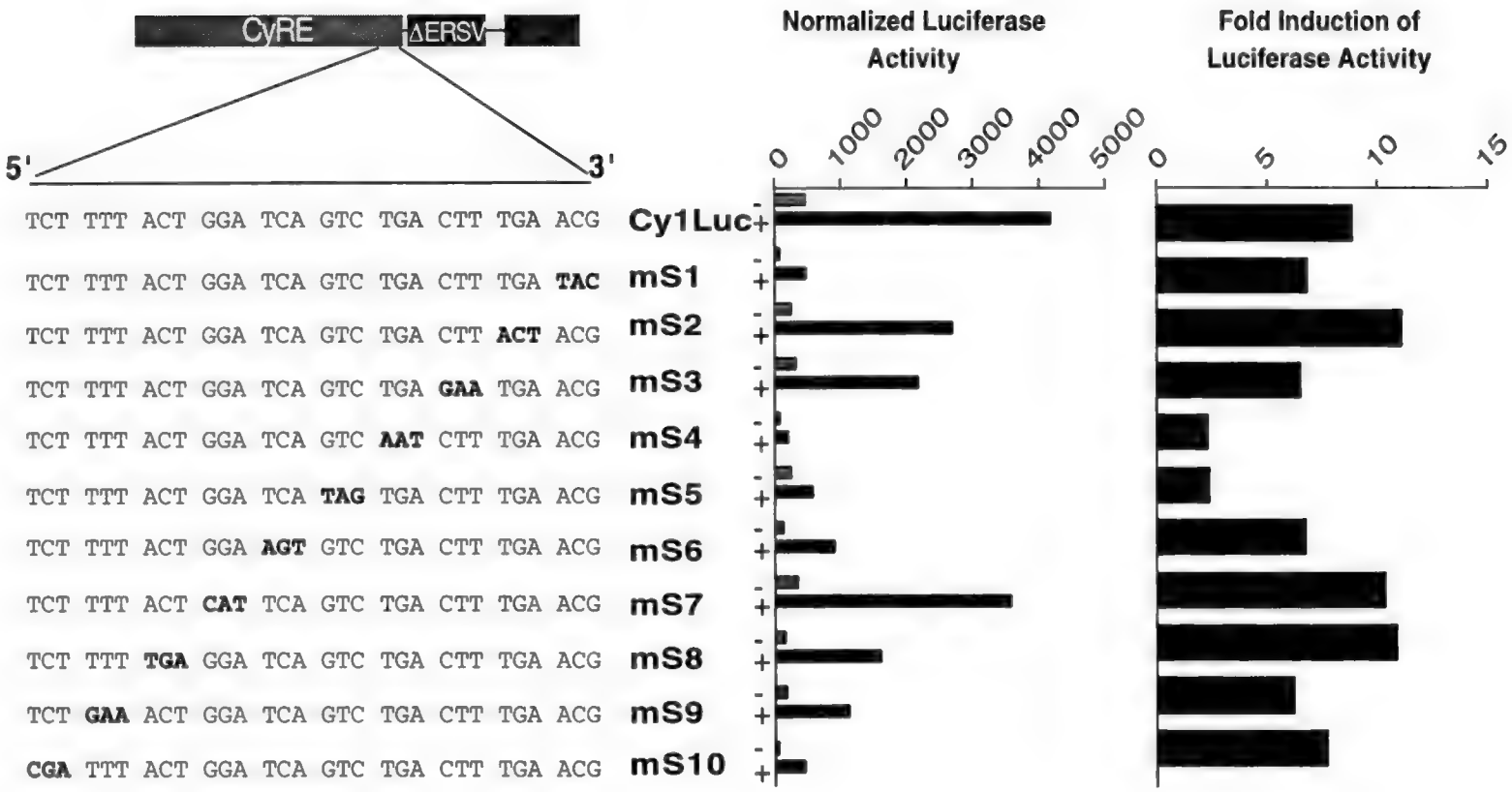
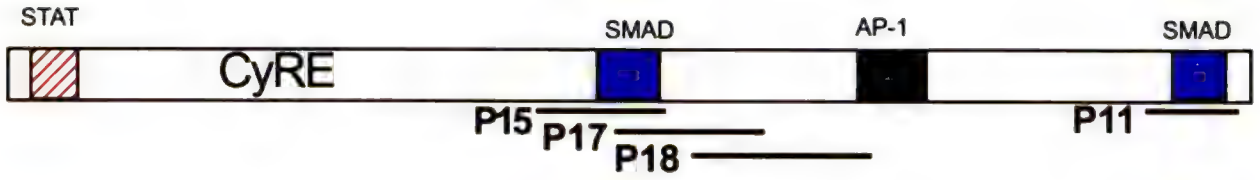

CyRE
ΔERSV
5'
3'
TCT TTT ACT GGA TCA GTC TGA CTT TGA ACG
TCT TTT ACT GGA TCA GTC TGA CTT TGA TAC
TCT TTT ACT GGA TCA GTC TGA CTT ACT ACG
TCT TTT ACT GGA TCA GTC TGA GAA TGA ACG
TCT TTT ACT GGA TCA GTC AAT CTT TGA ACG
TCT TTT ACT GGA TCA TAG TGA CTT TGA ACG
TCT TTT ACT GGA AGT GTC TGA CTT TGA ACG
TCT TTT ACT CAT TCA GTC TGA CTT TGA ACG
TCT TTT TGA GGA TCA GTC TGA CTT TGA ACG
TCT GAA ACT GGA TCA GTC TGA CTT TGA ACG
CGA TTT ACT GGA TCA GTC TGA CTT TGA ACG
Cy1Luc
mS1
mS2
mS3
mS4
mS5
mS6
mS7
mS8
mS9
mS10
Normalized Luciferase Activity
0
1000
2000
3000
4000
5000
Fold Induction of Luciferase Activity
0
5
10
15

Figure 14. Two distinct sites within the VIP CyRE are able to bind Smad transcription factors

EMSA was performed using various labeled regions of the CyRE and purified GST-ΔC Smad3 and GST-Smad4 proteins. Two distinct sites were able to bind these proteins: a region overlapped by P15 and P17 and a region contained in P11. Both proteins were able to bind to these sites although GST-ΔCSmad3 bound with a higher affinity. Both regions contain sequence homology with a consensus smad binding site. A labeled oligonucleotide of the Smad binding element (SBE) was used as the control.

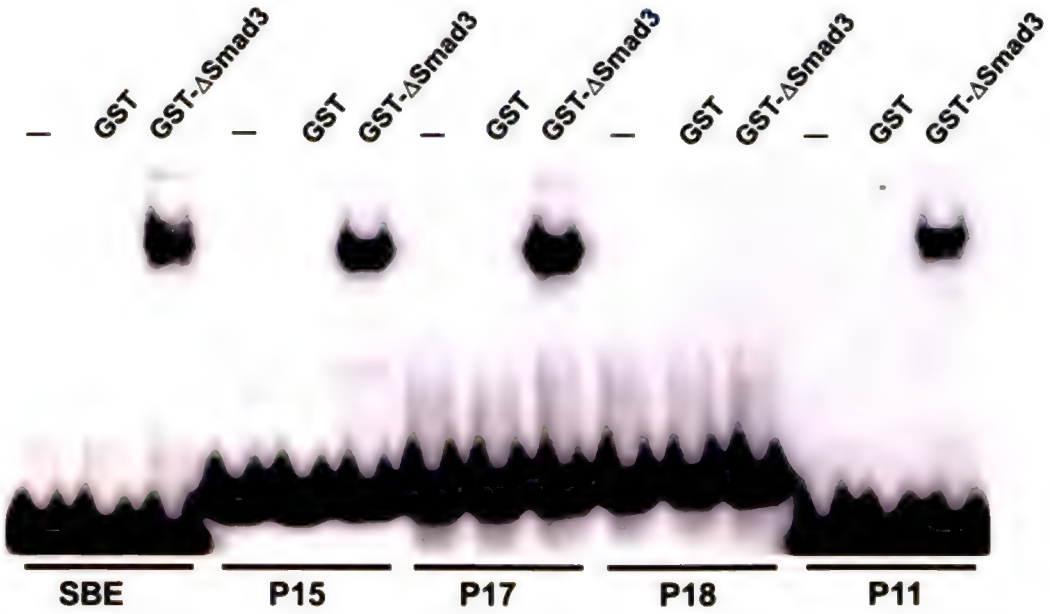


cgttcaaaGTCAGACTGatcca **P11**

gtacattgtcaGTCTAGACATact **SBE**

tcaactgggaaacaaattTCCAGACATtt **P15**

attTCCAGACATtttgaaacttaattcattta **P17**



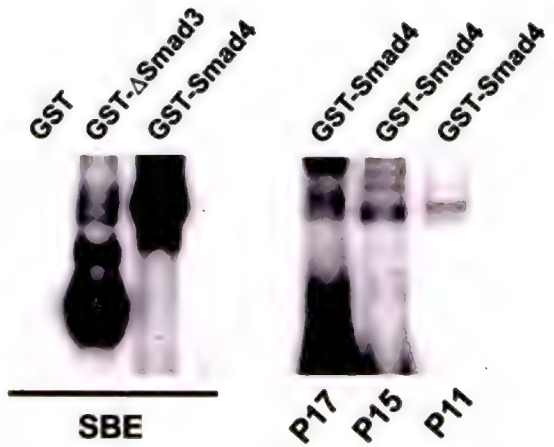


Figure 15. A truncated form of Smad 3 binds specifically to two sites in the VIP CyRE

EMSA was performed using purified GST-ΔCSmad3 and radiolabeled probes of P11, P15, and P17. Oligonucleotides mutated in the putative smad binding sites were synthesized and used as competitors in EMSAs. Competition of each probe with an excess unlabeled amount of itself and its mutant revealed that the binding seen with Smad 3 is specific. Competitions were performed with unlabelled 100 molar excess SBE and AP-1 oligonucleotides as positive and negative controls, respectively.

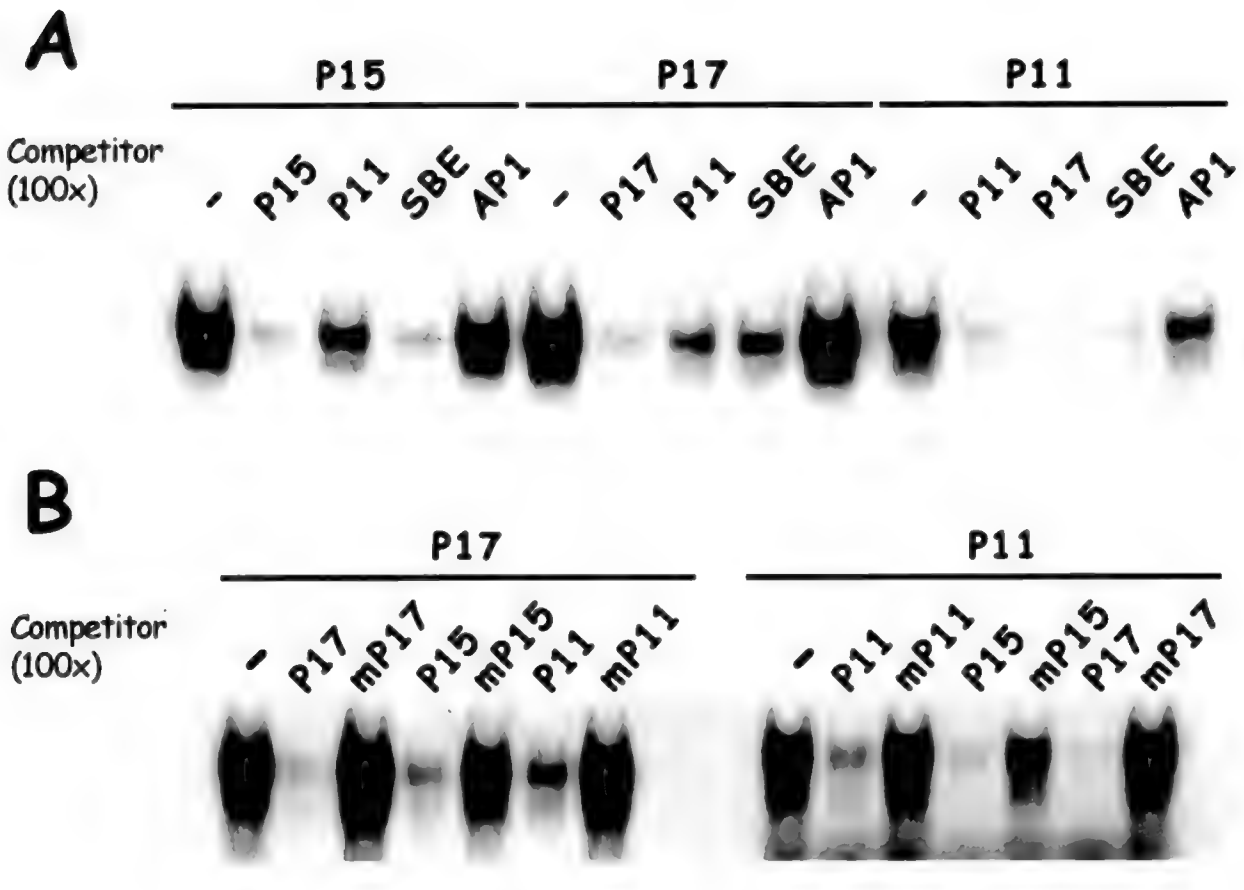
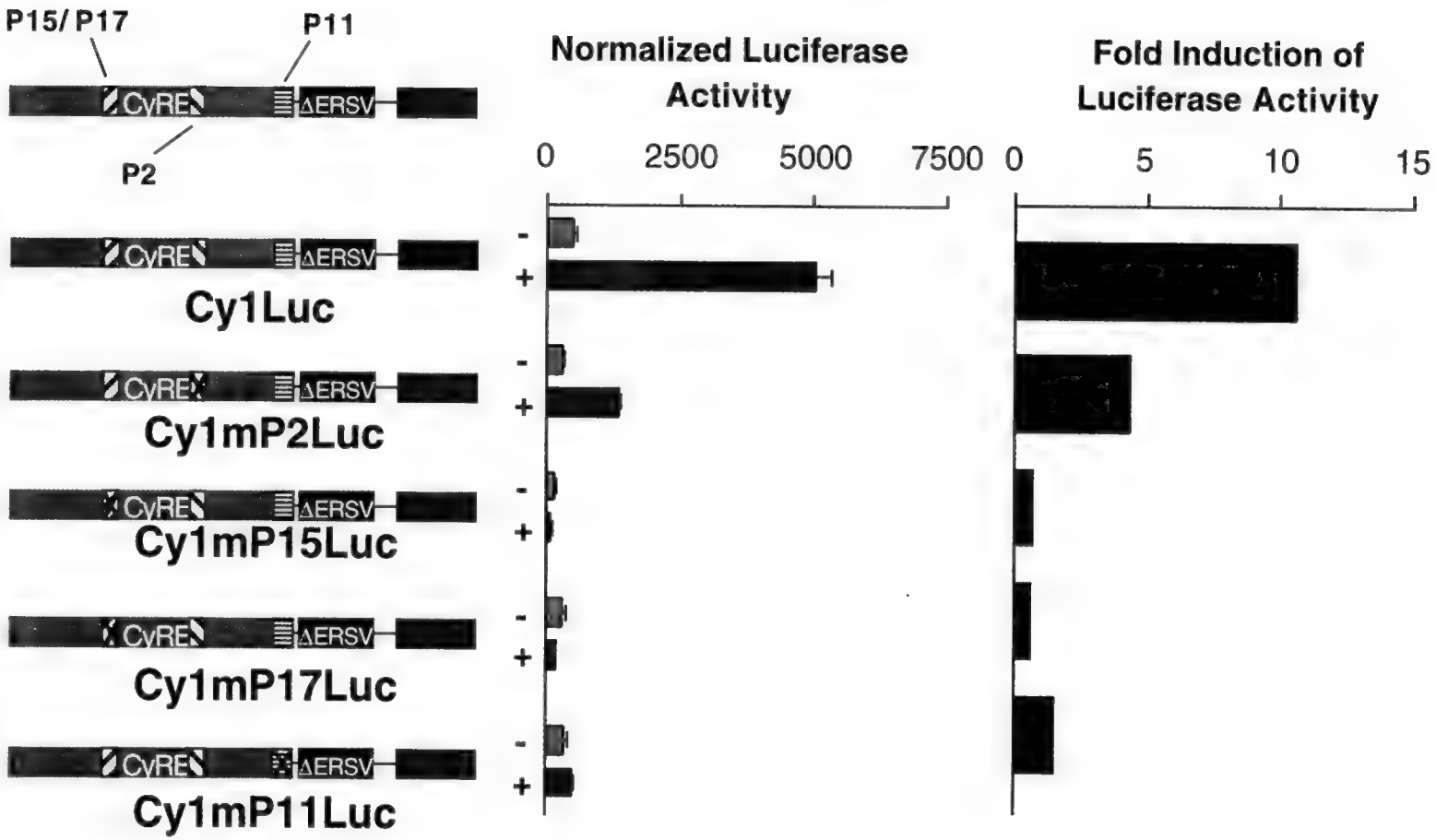

A
P15
P17
P11
Competitor
(100x)
-
P15
P11
SBE
AP1
-
P17
P11
SBE
AP1
-
P11
P17
SBE
AP1
B
P17
P11
Competitor
(100x)
-
P17
mP17
P15
mP15
P11
mP11
-
P11
mP11
P15
mP15
P17
mP17

Figure 16. TGF-β responsiveness is reduced by mutations within the CyRE Smad sites

NBFL cells, transfected with Cy1Luc plasmids mutated in transcription factor binding sites within the CyRE, were treated with (+) or without (-) TGF-β for 36 hours. The response to TGF-β was essentially abolished by mutations within the smad binding sites (Cy1mP15Luc, Cy1mP17Luc, or Cy1mP11Luc). Mutations within the AP-1 binding site (Cy1mP2Luc) saw a 60% reduction in TGF-β response. Luciferase values were normalized to the level of β-galactosidase expression.


P15/ P17
P11
CyRE
ΔERSV
P2
Cy1Luc
Cy1mP2Luc
Cy1mP15Luc
Cy1mP17Luc
Cy1mP11Luc
Normalized Luciferase Activity
0
2500
5000
7500
Fold Induction of Luciferase Activity
0
5
10
15
-
+

Discussion

There is growing evidence that expression of a single gene is often the result of tight regulation by multiple stimuli. To investigate regulation of the neuropeptide VIP gene by CNTF and TGF-β, we utilized a neuroblastoma cell line, NBFL, as a model for neural-crest derived peripheral neurons. NBFL cells are similar to peripheral neurons in that they regulate the expression of several neuropeptides in response to several different growth factors (Symes, et al., 1993; Tsokos, et al., 1997). Here we demonstrate that two members of very different cytokines families, CNTF and TGF-β, regulate the expression of vasoactive intestinal peptide (VIP) in NBFL cells.

Northern blot analysis of NBFL cells revealed that CNTF and TGF-β stimulate the expression of VIP mRNA (Figure 3). TGF-β treatment increased VIP mRNA levels less than CNTF treatment. Interestingly, co-treatment of NBFL cells with both cytokines resulted in a synergistic induction of VIP mRNA. Synergistic induction of VIP with CNTF was also demonstrated with Activin, a TGF-β superfamily member (Symes, et al., 2000).

Our data indicate that TGF-β induction of VIP is mediated through the cytokine response element (CyRE). TGF-β was able to induce transcription through the Cy1Luc construct that contains the entire 180-bp CyRE (Figure 5). This 180 bp element was initially characterized in CNTF regulation of VIP (Symes, et al., 1994). Since both cytokines utilize the CyRE to regulate VIP, known CNTF-mediated transcription factors may be possible targets of TGF-β signal transduction.

Two known transcriptional complexes bind to the CyRE after CNTF stimulation: a STAT-1/3 complex and a Fos/Jun complex (Symes, et al., 1997). While there have been no published reports of STAT-mediated signal transduction by TGF-β, there is much evidence that TGF-β induces AP-1 proteins and may sometimes induce gene transcription through AP-1 sites. Indeed, TGF-β has been demonstrated to be a regulator of c-Jun and JunB expression (Wong, et al., 1999; Jonk, et al., 1998; Li, et al., 1990; Pertovaara, et al., 1989). Regions within both c-Jun and JunB promoters have been identified as Smad3/Smad4 responsive sites that are critical for TGF-β mediated transcription (Wong, et al., 1999; Jonk, et al., 1998). In addition to regulation of AP-1 proteins themselves, the TGF-β mediated transcription factors, smads, also bind TPA-responsive elements to enhance AP-1 mediated transcription (Zhang, et al., 1998). This occurs through direct DNA binding of smads and through protein-protein interaction of smads with bound fos and jun members. This may explain why so many TGF-β responsive promotor elements also contain AP-1 binding sites (Wong, et al., 1999; Jonk, et al., 1998; Li, et al., 1990; Pertovaara, et al., 1989; Zhang, et al., 1998; Liberati, et al., 1999; Vindevoghel, et al., 1998). Thus our finding that TGF-β did not induce binding of AP-1 proteins to a consensus AP-1 oligonucleotide is surprising. The longest time point we examined with TGF-β treatment was 6 hours. Longer treatment times may be required to identify TGF-β activated AP-1 complexes. Its possible that TGF-β inducible AP-1 complexes are highly unstable and our EMSA conditions are not optimized for their binding to DNA. Northern blot analysis of fos and jun mRNA induction by TGF-β may also provide insight into TGF-β regulation of AP-1 proteins in NBFL cells. As we did

not detect TGF-β regulation of AP-1 proteins in NBFL cells, TGF-β involvement with AP-1 mediated transcription may be cell-line specific.

Smad proteins are a group of recently discovered transcription factors utilized by TGF-β superfamily members. TGF-β mediated signaling pathways phosphorylate and translocate smad proteins into the nucleus for transcriptional activation of target genes (Massague, 1998; Padgett, et al., 1998; Kretzschmar and Massague, 1998; ten Dijke, et al., 2000; Attisano and Wrana, 2000). TGF-β treatment of NBFL cells resulted in nuclear accumulation of either Smad 2 or Smad 3 within 15 minutes of treatment (Figure 9). TGF-β was able to induce transcription through a smad-responsive reporter, SBE-Luc, in NBFL cells (Figure 11). Both pieces of data suggest functional smad proteins are present in NBFL cells and are responsive to TGF-β.

TGF-β signal transduction results in the activation and heterodimerization of Smad3/Smad4 protein complexes. These complexes translocate into the nucleus where they activate gene transcription. Two putative smad binding sites were identified in the CyRE in the regions named P15/P17 and P11 (Figure 13). Both smad binding sites within the CyRE have sequence similarity to other known Smad 3 and Smad 4 binding sites (Dennler, et al., 1998; Jonk, et al., 1998; Song, et al., 1998). Characterization of the JunB promoter revealed a sequence (5'-AGACAAGGTTGT-3') capable of conferring TGF-β responsiveness and binding Smad 3 and Smad 4 (Jonk, et al., 1998). Regions of the PAI-1 promoter were also found to contain similar sequences (5'-CAGACA-3') capable of Smad 3 and Smad 4 binding (Dennler, et al., 1998; Jonk, et al., 1998). Both P15/P17 (5'-TCCAGACAT-3') and P11 (5'-GTCAGACTG-3') bind bacterially expressed Smad 4 and a truncated version of Smad 3 (Figure 14). Binding of Smad 4 to

P15/P17 and P11 was relatively weak compared to binding of truncated Smad 3, yet when bound to a control Smad Binding Element (SBE), Smad 4 binding equaled that of truncated Smad 3 (Figure 14). Thus, Smad 4 may require other DNA-binding proteins or co-activators to bind to the CyRE with higher affinity. Mutations in either P15/P17 or P11 site abrogated TGF-β inducible gene transcription through Cy1Luc (Figure 16). Thus the CyRE contains two consensus smad binding sites capable of binding truncated Smad 3 and Smad 4 and both sites are necessary for TGF-β mediated transcription.

Our data suggests that TGF-β regulation of VIP is mediated in part through smad proteins. Northern blot analysis revealed that simultaneous treatment of NBFL with CNTF and TGF-β synergistically induced VIP mRNA (Figure 3). We have established that TGF-β does not alter known CNTF-mediated STAT and AP-1 induction. Could CNTF possibly influence TGF-β mediated smad activation and translocation? CNTF was able to induce transcription 1.5 fold through Gal4-driven luciferase reporters when chimeric Gal4-Smad 2 proteins were transiently tranfected in NBFL cells (Figure 10). In contrast, CNTF was unable to induce transcription through the smad responsive reporter SBE-Luc (Figure 11). Thus, our data suggests CNTF is not a regulator of endogenous smad activation.

Although CNTF appears to not play a role in smad activation in NBFL cells, its role as a regulator of smad proteins in other systems in unclear. CNTF activates the MAP kinase signal transduction in multiple systems (Boulton, et al., 1994; Rajan and Mckay, 1998; Peterson, et al., 2000). Published reports have demonstrated the linker domain of Smad 3 as a site for phosphorylation by JNK (c-Jun N-terminal kinase), a member of the MAPK family (Engel, et al., 1999). JNK phosphorylation of Smad 3

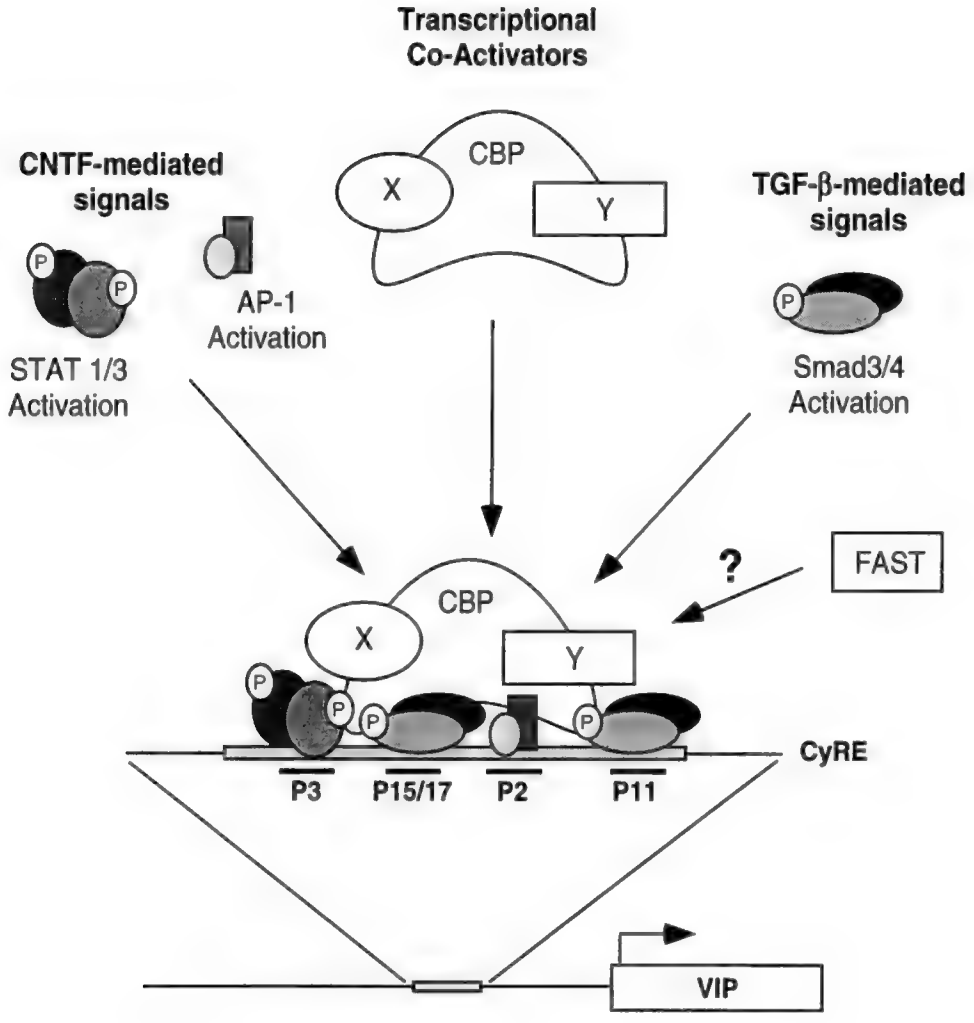
facilitated its activation and nuclear localization. Other MAPK family members may regulate Smad 3 in the same manner. Thus further investigation of CNTF-mediated MAPK activation and its role in Smad 3 regulation is needed.

Synergistic interaction between CNTF and TGF-β signaling pathways was demonstrated by northern blot analysis of VIP mRNA (Figure 3) and Cy1Luc-mediated transcription (Figure 5). CNTF appeared not to induce smad activation (Figures 10) and TGF-β appeared not to alter known CNTF-mediated pathways (Figures 7). Our data suggests that signals from both cytokines converge at the level of transcription factors binding to DNA. Although no direction interaction between proteins activated by either pathway was found, independent binding of smad proteins and STAT/AP-1 complexes to the CyRE was demonstrated (Figures 14 and 7, respectively). Smad homologues were demonstrated to bind to Activin responsive elements (ARE) along with FAST proteins in Xenopus and murine models (Chen, et al., 1997; Weisberg, et al., 1998; Yeo, et al., 1999). Interaction between CNTF and TGF-β signaling pathways may involve other DNA-binding proteins, such as FAST, for activation of gene transcription. Recently published reports identify CREB-binding protein (CBP) and its homologue, p300, as co-activators of smad-mediated transcription (Feng, et al., 1998; Topper, et al., 1998; Pouponnet, et al., 1998; Janknecht, et al., 1998; Nishihara, et al., 1998; Ghosh, et al., 2000). We hypothesize therefore that smads bind directly to the CyRE at specific sites and form larger transcriptional complexes with CNTF-activated STAT and AP-1 proteins through co-activators such as CBP. Further characterization of smad binding and possible smad-protein interactions on the CyRE is in progress.

Thus in a neuroblastoma cell line we demonstrated that TGF-β regulates VIP gene expression. TGF-β regulation of VIP is mediated through a responsive element also used by the gp130 cytokines to regulate VIP. TGF-β and CNTF use separate signaling cascades which appeared to function independently to mediate gene transcription. Interestingly, treatment with both cytokines resulted in a synergistic induction of gene transcription. The mechanism of this synergy is currently under investigation, but may result from the formation of a larger, more stable transcriptional complex enabling a much larger induction of VIP transcription when both cytokine signaling pathways are activated than by either alone. This complex may contain large co-activators such as CBP/p300 that may serve as a bridge between CNTF-mediated and TGF-β-mediated transcription factors. Such a complex may serve as a novel mechanism of VIP regulation.

Figure 17. Model of TGF-β and CNTF signaling to the CyRE in NBFL cells

A proposed model of TGF-β and CNTF synergy is shown. CNTF utilizes the JAK/STAT pathway for its signal transduction. Upon receptor activation, associated Janus Kinases transphosphorylate each other. Phosphorylated JAKs then phosphorylate tyrosine residues along the cytoplasmic tail of the opposite receptor component. These phosphotyrosine moieties then become docking sites for STAT proteins. Docked STATS become phosphorylated and form heterodimers with each other and translocate into the nucleus. There they bind to the specific STAT-binding site known as P3. Also ligand binding to receptor leads to activation of other kinase cascades resulting in the formation of an AP-1 complex. This is most likely formed in the nucleus where the AP-1 complex binds to the specific AP-1 binding site, P2. We now think that TGF-β receptor activation leads to the formation of a Smad complex in NBFL cells. Receptor activation leads to Smad 3 phosphorylation and complex formation with the co-smad, Smad 4. This complex then translocates to the nucleus where it binds to two sites within the CyRE, P15/P17 and P11, possibly in conjunction with other binding proteins. CNTF activation of STAT and AP-1 proteins together with TGF-β activation of the smad pathway leads to a very robust activation of VIP transcription. We hypothesize that this strong activation is due to the formation of a larger transcriptional complex on the CyRE composed of basal, cell-specific, and cytokine-activated transcription factors bridged by co-activators. Thus, simultaneous stimulation of two distinct cytokine signaling pathways converge to regulate synergistically VIP gene transcription.


Transcriptional
Co-Activators
CBP
X
Y
CNTF-mediated
signals
AP-1
Activation
TGF-β-mediated
signals
STAT 1/3
Activation
Smad3/4
Activation
?
FAST
CBP
X
Y
CyRE
P3
P15/17
P2
P11
VIP

Bibliography

Alam, J. and Cook, J. L. (1990) Reporter genes: application to the study of mammalian gene transcription. Anal. Biochem. **188**, 245-254.

Attisano, L. and Wrana, J. L. (2000) Smads as transcriptional co-modulators. Curr. Opin. Cell Biol. **12**, 235-243.

Bannister, J. A., and Kouzarides, T. (1996) The CBP co-activator is a histone acetyltransferase. Nature **384**, 641-643.

Barbin, G., Manthorpe, M., and Varon, S. (1984) Purification of the chick eye ciliary neurotrophic factor. J. Neurochem. **43**, 1468-1478.

Baumann, H., Symes, A. J., Comeau, M. R., Morella, K. K., Wang, Y., Friend, D., Ziegler, S. F., Fink, J. S., and Gearing, D. P. (1994) Multiple regions within the cytoplasmic dominas of the leukemia inhibitory factor receptor and gp130 cooperate in signal transduction in hepatic and neuronal cells. Mol. Cell. Biol. **14**, 138-146.

Bazan, J. F. (1991) Neuropoietic cytokines in the hematopoietic fold. Neuron **7**, 197-208.

Boulton, T. G., Stahl, N., and Yancopoulos G. D. (1994) Ciliary neurotrophic factor/leukemia inhibitory factor/interleukin 6/oncostatin M family of cytokines induces tyrosine phosphorylation of a common set of proteins overlapping those induced by other cytokines and growth factors. J. Biol. Chem. **269**, 11648-11655.

Boyd, F. T. and Massague, J. (1989) Transforming growth factor-beta inhibition of epithelial cell proliferation linked to the expression of a 53-kDa membrane receptor. J. Biol. Chem. **264**, 2272-2278.

Chen, X., Weisberg, E., Fridmacher, V., Watanabe, M., Naco, G., and Whitman, M. (1997) Smad 4 and FAST-1 in the assembly of Activin-responsive factor. Nature **389**, 85-89.

Davis, S., Aldrich, T. H., Valenzuela, D. M., Wong, V. V., Furth, M. E., Squinto, S. P., and Yancopoulos, G. D. (1991) The receptor for ciliary neurotrophic factor. Science **253**, 59-63.

de Wet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R., and Subramani, S. (1987) Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. **7**, 725-737.

Engel, M. E., McDonnell, M. A., Law, B. K., and Moses, H. L. (1999) Interdependent SMAD and JNK signaling in transforming growth factor-β-mediated transcription. J. Biol. Chem. **274**, 37413-37420.

Feng, J., Witthuhn, B. A., Matsuda, T., Kohlhuber, F., Kerr, I. M., and Ihle, J. N. (1997) Activation of JAK2 catalytic activity requires phosphorylation of Y1007 in the kinase activation loop. Mol. Cell. Biol. **17**, 2497-2501.

Feng, X.-H., Zhang, Y., Wu, R.-Y., and Derynck, R. (1998) The tumor suppressor Smad 4/DPC4 and transcriptional adaptor CBP/p300 are coactivators for smad 3 in TGF-β-induced transcriptional activation. Genes Dev. **12**, 2153-2163.

Franzen, P., ten Dijke, P., Ichijo, H., Yamashita, H., Schultz, P., Heldin, C. H., and Miyazono, K. (1993) Cloning of a TGF beta type I receptor that forms a heteromeric complex with the TGF beta type II receptor. Cell **75**, 681-692.

Gauzzi, M. C., Valezquez, L., McKendry, R., Mogensen, K. E., Fellous, M., and Pellegrini, S. (1996) Interferon-α-dependent activation of Tyk2 requires phosphorylation of positive regulatory tyrosines by another kinase. J. Biol. Chem. **271**, 20494-20500.

Gearing, D. P. and Bruce, A. G. (1992) Oncostatin M binds the high-affinity leukaemia inhibitory factor receptor. New Biol. **4**, 61-65.

Gearing, D. P., Comeau, M. R., Friend, D. J., Gimpel, S. D., Thut, D. J., McGourty, J., Brasher, K. K., King, J. A., Gillis, S., and Mosley, B. et al. (1992) The IL-6 signal transducer. gp130- an oncostatin M receptor and affinity converter for the LIF receptor. Science **255**, 1434-1437.

Gearing, D. P., Gough, N. M., King, J. A., Hilton, D. J., Nicola, N. A., Simpson, R. J., Nice, E. C., Kelso, A., and Metcalf, D. (1987) Molecular cloning and expression of cDNA encoding a muring myeloid leukaemia inhibitory factor (LIF). EMBO J. **6**, 3995-4002.

Ghosh, A. K., Yuan, W., Mori, Y., and Varga, J. (2000) Smad-dependent stimulation of type I collagen gene expression in human skin fibroblasts by TGF-beta involves functional cooperation with p300/CBP transcriptional coactivators. Oncogene **19**, 3546-3555.

Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**, 1044-1051.

Heim, M. H. (1999) The Jak-STAT pathway: cytokine signalling from the receptor to the nucleus. J. Recept. Signal Transduct. Res. **19**, 75-120.

Imada, K. and Leonard, W. J. (2000) The Jak-STAT pathway. Mol. Immuno. **37**, 1-11.

Ip, N. Y., McClain, J., Barrezueta, N. X., Aldrich, T. H., Pan, L., Li, Y., Wiegand, S. J., Friedman, B., Davis, S., and Yancopoulos, G. D. (1993) The alpha component of the CNTF receptor is required for signaling and defines potential CNTF targets in the adult and during development. Neuron **10**, 89-102.

Ip, N. Y., Nye, S. H., Boulton, T. G., Davis, S., Taga, T., Li, Y., Birren, S. J., Yasakawa, K., Kishimoto, T., Anderson, D. J., Stahl, N., and Yancopoulos, G. D. (1992) CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp-130. Cell **69**, 1121-1132.

Janknecht, R., Wells, N. J., and Hunter, J. (1998) TGF-β-stimulated cooperation of smad proteins with the coactivators CBP/p300. Genes Dev. **12**, 2114-2119.

Jones, E. A., Conover, J., and Symes, A. J. (2000) Identification of a novel gp130 responsive site in the vasoactive intestinal peptide cytokine response element. J. Biol. Chem. (in press)

Jonk, L. J. C., Itoh, S., Heldin, C.-H., ten Dijke, P., and Kruijer, W. (1998) Identification and functional characterization of a smad binding element (SBE) in the

JunB promoter that acts as a transforming growth factor-β, activin, and bone morphogenetic protein-inducible enhancer. J. Biol. Chem. **273**, 21145-21152.

Kretzschmar, M. and Massague, J. (1998) Smads: mediators and regulators of TGF-β signaling. Curr. Opin. Genet. Dev. **8**, 103-111.

Laiho, M., Weis, M. B., and Massague, J. (1990) Concomitant loss of transforming growth factor (TGF)-beta receptor types I and II in TGF-beta-resistant cell mutants implicates both receptor types in signal transduction. J. Biol. Chem. **265**, 18518-18524.

Leung, D. W., Parent, A. S., Cachianes, G., Esch, F., Coulombe, J. N., Nikolics, K., Eckenstein, F. P., and Nishi, R. (1992) Cloning, expression during development, and evidence for release of a trophic factor for ciliary ganglion neurons. Neuron **8**, 1045-1053.

Li, L., Hu, J.-S., and Olson, E. N. (1990) Different members of the jun proto-oncogene family exhibit distinct patterns of expression in response to type β transforming growth factor. J. Biol. Chem. **265**, 1556-1562.

Liberati, N. T., Datto, M. B., Frederick, J. P., Shen, X., Wong, C., Rougier-Chapman, E. M., and Wang, X.-F. (1999) Smads bind directly to the Jun family of AP-1 transcription factors. Proc. Natl. Acad. Sci. USA **96**, 4844-4849.

Lin, L. F., Mismer, D., Lile, J. D., Armes, L. G., Butler, E. B., Vannice, J. L., and Collins, F. (1989) Purification, cloning, and expression of ciliary neurotrophic factor (CNTF). Science **246**, 1023-1025.

Liu, K. D., Gaffen, S. L., and Goldsmith, M. A. (1998) JAK/STAT signaling by cytokine receptors. Curr. Opin. Immuno. **10**, 271-278.

Liu, K. D., Gaffen, S. L., Goldsmith, M. A., and Greene, W. C. (1997) Janus kinases in interleukin-2-mediated signaling: JAK1 and JAK3 are differentially regulated by tyrosine phsophorylation. Curr. Biol. **7**, 817-826.

Luo, K., Stroschein, S. L., Wang, W., Chen, D., Martens, E., Zhou, S., and Zhou, Q. (1999) The ski oncoprotein interacts with the smad proteins to repress TGFβ signaling. Genes & Dev. **13**, 2196-2206.

Malik, N., Kallestad, J. C., Gunderson, N. L., Austin, S. D., Neubauer, M. G., Ochs, V., Marguardt, H., Zarling, J. M., Shoyah, M. and Wei, C. M., et al. (1989) Molecular cloning, sequence analysis, and functional expression of a novel growth regulator, oncostatin M. Mol. Cell. Biol. **9**, 2847-2853.

Massague, J. (1998) TGF-β signal transduction. Ann. Rev. Biochem. **67**, 753-791.

Murphy, M., Dutton, R., Koblar, S., Cheema, S., and Bartlett, P. (1997) Cytokines which signal through the LIF receptor and their actions in the nervous system. Prog. Neurobiol. **52**, 355-378.

Nishihara, A., Hanai, J.-I., Imamura, T., Miyazono, K., and Kawabata, M. (1999) E1A inhibits transforming growth factor-β signaling through binding to smad proteins. J. Biol. Chem. **274**, 28716-28723.

Nomura, T., Khan, M. M., Kaul, S. C., Dona, H.-D., Wadhwa, R., Colmenares, C., Kohno, I., and Ishii, S. (1999) Ski is a component of the histone deacetylase complex required for transcriptional repression by Mad and thyroid hormone receptor. Genes & Dev. **13**, 412-423.

Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell **87**, 953-959.

Padgett, R. W., Cho, S.-H., and Evangelista, C. (1998) Smads are the central component in transforming growth factor-β signaling. Pharmacol. Ther. **78**, 47-52.

Pennica, D., King, K. L., Shaw, K. J., Luis, E., Rullamas, J., Luoh, S. M., Darbonne, W. C., Knutzon, D. S., Yen, R., and Chien, K. R., et al. (1995) Expression cloning of

cardiotrophin 1, a cytokine that induces cardic myocyte hypertrophy. Proc. Natl. Acad. Sci. USA **92**, 1142-1146.

Pertovaara, L., Sistonen, L., Bos, T. J., Vogt, P. K., Keski-Oja, J., and Alitalo, K. (1989) Enhanced jun gene expression in an early genomic response to transforming growth factor β stimulation. Mol. Cell. Biol. **9**, 1255-1262.

Peterson, W. M., Wang, Q., Tzekova, R., and Wiegand, S. J. (2000) Ciliary neurotrophic factor and stress stimuli activate the Jak-STAT pathway in retinal neurons and glia. J. Neurosci. **20**, 4081-4090.

Pouponnot, C., Jayaraman, L., and Massague, J. (1998) Physical and functional interaction of smads and p300/CBP. J. Biol. Chem. **273**, 22865-22868.

Rajan, P. and McKay, R. D. (1998) Multiple routes to astrocytic differentiation in the CNS. J. Neurosci. **18**, 3620-3629.

Segal, R. A. and Greenberg, M. E. (1996) Intracellular signaling pathways activated by neurotophic factors. Ann. Rev. Neurosci. **19**, 463-489.

Sleeman, M. W., Anderson, K. D., Lambert, P. D., Yancopoulos, G. D., and Wiegand, S. J. (2000) The ciliary neurotrophic factor and its receptor, CNTFR alpha. Pharm. Acta. Helv. **74**, 265-272.

Souchelnytskyi, S., ten Dijke, P., Miyazono, K., and Heldin, C. H. (1996) Phosphorylation of Ser165 in TGF-beta type I receptor modulates TGF-beta1-induced cellular responses. EMBO J. **15**, 6231-6240.

Stahl, N., Boulton, T. G., Farruggella, T., Ip, N. Y., Davis, S., Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Barbieri, G., Pellegrini, S., Ihle, J. N., and Yancopoulos, G. D. (1994) Association and activation of JAK TYK kinases by CNTF-LIF-OSM-IL-6 beta-receptor components. Science **263**, 92-95.

Stahl, N. and Yancopoulos, G. D. (1994) The tripartite CNTF receptor complex: activation and signaling involves components shared with other cytokines. J. Neurobiol. **25**, 1454-1466.

Symes, A. J., Mahendra, S. R., Lewis, S. E., Landis, S. C., Hyman, S. E., and Fink, J. S. (1993) Ciliary neurotrophic factor coordinately activates transcription of neuropeptide genes in a neuroblastoma cell line. Proc. Natl. Acad. Sci. USA **90**, 572-576

Symes, A., Lewis, S., Corpus, L., Rajan, P., Hyman, S., and Fink, J. S. (1994) STAT proteins participate in the regulation of the vasoactive intestinal peptide gene by the ciliary neurotrophic factor family of cytokines. Mol. Endocrinol. **8**, 1750-1763.

Symes, A. J., Corpus, L., and Fink, J. S. (1995) Differences in nuclear signaling by leukemia inhibitory factor and interferon-γ: The role of STAT proteins in regulating vasoactive intestinal peptide gene expression. J. Neurochem. **65**, 1926-1933.

Symes, A., Gearan, T., Eby, J., and Fink, J. S. (1997) Interaction of Jak-Stat and AP-1 signaling pathways at the vasoactive intestinal peptide cytokine response element regulates ciliary neurotrophic factor-dependent transcription. J. Biol. Chem. **272**, 9648-9654.

Symes, A. J., Pitts, R. L., Conover, J., Kos, K., and Coulombe, J. Synergy of Activin and ciliary neurotrophic factor signaling pathways in the induction of vasoactive intestinal peptide gene expression. Mol. Endo. **14**, 429-439.

ten Dijke, P., Miyazono, K., and Heldin, C.-H. (2000) Signaling inputs converge on nuclear effectors in TGF-β signaling. TIBS **25**, 64-70.

Topper, J. N., DiChiara, M. R., Brown, J. D., Williams, A. J., Falb, D., Collins, T., and Gimbrone, Jr., M. A. (1998) CREB binding protein is a required coactivator for smad-depenedent, transforming growth factor β transcriptional responses in endothelial cells. Proc. Natl. Acad. Sci. USA **95**, 9506-9511.

Tsokos, M., Scarpa, S., Ross, R. A., and Triche, T. J. (1987) Differentiation of human neuroblastoma recapitulates neural crest development. Study of morphology,

neurotransmitter enzymes, and extracellular matrix proteins. Am. J. Pathol. **128**, 484-496.

Tsukazaki, T., Chiang, T. A., Davison, A. F., Attisano, L., and Wrana, J. L. (1998) SARA, a FYVE domain protein that recruits Smad 2 to the TGF-β receptor. Cell **95**, 779-791.

Weisberg, E., Winnier, G. E., Chen, X., Farnsworth, C. L., Hogan, B. L., and Whitman, M. (1998) A mouse homologue of FAST-1 transduces TGF beta superfamily signals and is expressed during early embryogenesis. Mech. Dev. **79**, 17-27.

Wieser, R., Wrana, J. L., and Massague, J. (1995) GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex. EMBO J. **14**, 2199-2208.

Wong, C., Rougier-Chapman, E. M., Frederick, J. P., Datto, M. B., Liberati, N. T., Li, J-M., and Wang, X-F. (1999) Smad3-Smad4 and AP-1 complexes synergize in transcriptional activation of the c-jun promoter by transforming growth factor β. Mol. Cell. Biol. **19**, 1821-1830.

Wood, K. V. (1990) Firefly luciferase: a new tool for molecular biologists. Promega Notes **28**, 1.

Wotton, D., Lo, R. S., Lee, S., and Massague, J. (1999) A smad transcriptional corepressor. Cell **97**, 29-39.

Wrana, J. L., Attisano, L., Wieser, R., Ventura, F., and Massague, J. (1994) Mechanism of activation of the TGF-beta receptor. Nature **370**, 341-347.

Verschueren, K., Remacle, J. E., Collart, C., Kraft, H., Baker, B. S., Tylzanowski, P., Nelles, L., Wuytens, G., Su, M.-T., Bodmer, R., Smith, J. C., and Huylebroeck, D. (1999) SIP1, a novel zinc finger/homeodomain repressor, interacts with smad proteins and binds to 5'-CACCT sequences in candidate target genes. J. Biol. Chem. **274**, 20489-20498.

Vindevoghel, L., Kon, A., Lechleider, R. J., Uitto, J., Roberts, A. B., and Mauviel, A. (1998) Smad-dependent transcriptional activation of human type VII collagen gene (COL7A1) promoter by transforming growth factor-β. J. Biol. Chem. **273**, 13053-13057.

Yeo, C. Y., Chen, X., and Whitman, M. (1999) The role of FAST-1 and smads in transcriptional regulation by activin during early Xenopus embryogenesis. J. Biol. Chem. **274**, 26584-26590.

Zhang, Y., Feng, X.-H., and Derynck, R. (1998) Smad 3 and Smad 4 cooperate with c-jun/c-fos to mediate TGF-β-induced transcription. Nature **394**, 909-913.

Zhang, Y. and Derynck, R. (1999) Regulation of smad signaling by protein associatiosn and signaling crosstalk. Trends Cell Biol. **9**, 274-279.